Towards an understanding of the mechanism of heredity / H.L.K. Whitehouse ; foreword by G. Pontecorvo.

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Towards an Understanding of the Mechanism of Heredity

"How odd it is that anyone should not see that all observation must be for or against some view if it is to be of any service!"

Charles Darwin, 18 September 1861, in a letter to Henry Fawcett. (*More Letters of Charles Darwin*, edited by Francis Darwin and A. C. Seward (1903). London, John Murray, vol. 1, pp. 194–196.)

Fawcett had just addressed the Botany and Zoology Section at the Manchester Meeting of the British Association for the Advancement of Science 'on the Method of Mr. Darwin in his Treatise on the Origin of Species'. In the discussion that followed, Edwin Lankester expressed the opinion that the facts which Darwin had brought forward in support of the hypothesis of evolution by natural selection were of more value than the hypothesis itself. This gave rise to the remark in Darwin's letter.

Towards an Understanding of the Mechanism of Heredity

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To my MOTHER and FATHER
who first aroused my interest in the
scientific study of plants and animals

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Foreword

Genetics—the study of heredity and variation—is going through a phase of obvious success. It is inevitable that a proportion of the young and more able artificers of this success should have come to think that what matters in genetics dates, say, from 1953. That was the time of the remarkable elucidation by Watson and Crick of the structure of DNA with its all embracing implications. In the last two years quite a number of books, some of them admirable, have been written with that conscious or unconscious attitude.

Dr. Whitehouse's book is refreshingly different. It traces the basic ideas of Genetics through their development and describes concisely, yet without omitting any essential detail, the critical experiments which led to the establishment of each of those ideas. This historical approach, invaluable for teachers and no doubt appealing to the more thoughtful students, is not at the expense of keeping abreast with the advancing frontiers. Suffice it to say that in one chapter Dr. Whitehouse deals with the latest genetical and biochemical approaches to recombination—a stagnant field since about 1930—which gives hope that this basic biological process may soon become understood.

To the honour of having been asked to write this foreword I can add the pleasure of having been compelled to read a most valuable book.

Glasgow
September, 1965

G. Pontecorvo

Preface to the First Edition

This publication is intended as an account of the primary evidence for current beliefs concerning the mechanism of heredity. Other aspects of genetics are referred to only if their study has illuminated fundamental features of heredity. Thus, the book deals only with the central core of genetics, omitting the more peripheral components. There is no discussion of how the hereditary mechanism may have evolved, nor of how evolutionary divergence of populations and species may have come about, and discussion of the many applications of genetics, such as to medicine, and to crop and livestock improvement, is omitted. The aspects of genetics which are included occupy such a central position in biology that in varying degrees all the rest of biology may be said to be related to them.

The theme of the book has been to trace the development of ideas about heredity from the earliest times. The approach, however, is not strictly historical. Each of the 16 chapters deals with a particular hypothesis concerning a fundamental feature of the hereditary mechanism, and sets out to show how experimental evidence has either confirmed the theory, or led to its modification or its abandonment. The classic experiments which gave rise to major advances are described, together with the inferences drawn from them. Particular trouble has been taken to distinguish observation from deduction. In this way it is hoped that the nature of the scientific method will be clearly demonstrated. The chemical and physical evidence for the structure of the components of DNA (Chapter 11), and the evidence from biochemical studies for the steps in protein synthesis (Chapter 14), are not given. On the other hand, some idea of the nature of the evidence for all the conclusions reached about the hereditary mechanism is included, with the exception of some of the inferences about genetic recombination in bacteria. Some features of the study of the hereditary mechanism are rather complex, but no attempt has been made to omit them on that account. In consequence, certain parts of the book are not easy reading, for instance, §§ 4.8, 8.6 and 9.8.

The book is intended for any interested person who has some background knowledge of biology and chemistry. No attempt has been made to describe the structure or life-cycle of the organisms mentioned in the text, but a systematic list of them is included (Appendix 1). It is hoped that the book will be useful to Biology students at Universities, including research students. Much of the book is based on the courses of lectures in genetics which I have given to first-, second- and third-year students reading Botany at Cambridge University. A glossary of genetical terms is included (Appendix

2) for the benefit of readers unfamiliar with the subject, and an extensive bibliography of original papers for those wishing to obtain more detailed information about the topics discussed.

The book has been written in the hope that an account of the remarkable progress that has been made (beginning with Mendel just a century ago) in understanding the mechanism of heredity, will give some readers the pleasure of intellectual stimulation. The immense advances of the last 20 years have greatly illuminated many of the earlier discoveries by revealing their molecular basis, and I hope readers will be able to recapture something of the intellectual excitement that this has caused. Much of the basic fabric of living organisms now stands revealed, with its central enigma of how the extraordinary interlocked system of nucleic acids and proteins (both of which, in different ways, are necessary for the synthesis of each) first evolved.

I wish to thank all those who have helped me in the preparation of this book. Dr. D. Briggs, Mr. M. H. V. Cooray, Dr. S. A. Henderson and Dr. G. Meyer have provided photographs, and I am indebted to them for their generosity. I am grateful to Dr. Sydney Smith for tracing the source of the Darwin quotation on p. (ii), and I owe a special acknowledgement to Professor G. Pontecorvo, F.R.S., for his kindness in writing the Foreword. I would like also particularly to thank Mr. G. J. Clark for the substantial help he has given me in the preparation of many of the diagrams. Mrs. Pamela Landshoff kindly typed part of the manuscript, and Mr. F. T. N. Elborn took the photographs reproduced as Plate 4 (b)–(e). I thank Messrs. Oliver and Boyd for permission to publish Table 4.2. In thanking my Publishers, I should like to mention especially their unfailing courtesy and helpfulness. Finally, I would express my gratitude to Pat, my wife, not only for the assistance she has given me, but also for putting up with my many hours of study.

Cambridge
February, 1965

H. L. K. Whitehouse

Preface to the Second Edition

In revising the book to take account of the remarkable developments in the last three years, I have largely rewritten the last three chapters, dividing the final one into three numbered 15, 17 and 18, the former Chapter 15 being renumbered 16. Some changes have also been made in Chapter 12 and slight alterations to several others. Over 250 references have been added to the bibliography.

I am grateful to Dr T. Butterfass of the Max-Planck-Institut für Pflanzengenetik at Ladenburg, Dr J. A. Hunt of the Department of Genetics, University of Hawaii, and Dr N. W. Simmonds, Director of the Scottish Plant Breeding Station, for drawing my attention to errors in the previous edition. I thank Dr S. A. Henderson of the Department of Genetics, University of Cambridge, for helpful discussions about subchromatid exchanges. Some of the revision of the book was carried out while I was a member of the Faculty of the Division of Biology at the Southwest Center for Advanced Studies at Dallas, Texas, and I would like to thank Dr Carsten Bresch and other colleagues for the opportunity to discuss many questions in genetics.

Cambridge
August, 1968

H. L. K. Whitehouse

Contents

1. *The classical theory of direct inheritance of characters*

§ 1.1 The ideas of Hippocrates

Among the earliest known writings on the subject of heredity are those of Hippocrates (ca. 400 B.C.). He believed that the reproductive material came from all parts of the individual's body and hence that characters were directly handed down to the progeny. As evidence in support of this hypothesis he referred to a race of mankind called the Macrocephali, who, immediately after a child was born, fashioned its head by hand to give it an elongated shape. He also said that, at a later period, the elongated head was formed naturally, without the necessity of moulding it after birth. Hippocrates asked why, since such characters as baldness and blue eyes are inherited, should not the long-headed character be similarly handed down. Since he believed that the reproductive material coming from unhealthy parts of the body was correspondingly unhealthy, he was willing to accept the notion that mutilations, such as the moulding of the head, would in the course of one or a few generations become inherited. Hippocrates must have relied on hearsay for supporting his theory of heredity, as he could hardly have made direct observations of a change in a human population over a number of generations.

§ 1.2 The views of Aristotle

Aristotle (ca. 350 B.C.) questioned the Hippocratic view and pointed out the difficulties, even impossibilities, which it presented. He referred to the inheritance of such characters as the voice, nails, hair, and way of moving, which he believed could not contribute to the reproductive material, since they were intangible or concerned dead tissues, and he also referred to characters such as a beard or grey hair which may not be present at the time of reproduction. Further, Aristotle pointed out that children may resemble a grandparent more closely than they resemble their parents. He also drew attention to the dilemma which the Hippocratic theory presents when applied to plants, where, for various reasons, a part may be absent at the times of reproduction and yet be inherited. He was puzzled, too, as to how the two parents could each contribute something from all their parts, and yet their progeny have but one and not two of each part.

Aristotle argued that the reproductive material, instead of being derived

from all the parts of the individual as Hippocrates thought, should be regarded as made up of nutrient substances which, while on their way to the various parts, had been diverted to the reproductive path. These nutrient substances would differ from one another depending on the part of the organism for which they were originally destined. He also favoured the idea that the contributions to the progeny from the two sexes were not the same, the female contributing the material and the male something to define the form that the embryo is to assume, just as the carpenter's contribution to the making of a chair is merely its shape and form.

The ideas of Hippocrates and of Aristotle concerning heredity differ, but share the notion that inheritance is direct: that is, that substances derived from or intended for specific parts of the individual are handed down to the progeny. Hypotheses of this kind represent the simplest theory of heredity which one can postulate.

§ 1.3 Darwin's theory

Over the centuries many variations of the theories of Hippocrates and Aristotle have been proposed, but the basic idea that characters are in some way transmitted directly from parent to offspring was never effectively challenged until the year 1883 when Weismann proposed his theory of the continuity of the germ-plasm. Mendel's conclusions (1866) implied the discarding of the classical view of heredity, but this was not appreciated at the time, as his contribution was largely overlooked until 1900. Thus, Darwin (1868, Chapter 27) suggested that all the cells and tissues of an organism threw off minute granules, both during development and when the individual had reached maturity. He further supposed that these granules circulated through the plant or animal, multiplied, and were passed to the reproductive cells, which thus contained a multitude of components thrown off from each individual part of the organism. In this way, hereditary characters were thought to be transmitted to the progeny. In the offspring the granules were regarded as responsible for the development of cells or tissues corresponding to those from which they had been derived in the parent. The granules were supposed to be transmitted occasionally in a dormant state for several generations before developing to reveal an ancestral character. As Darwin (1875, Chapter 27, footnote 42) himself remarked, this theory of heredity resembles that of Hippocrates. It differs chiefly in specifying that granules were responsible for the hereditary transmission.

§ 1.4 Experimental results obtained by Knight and by Goss

Experimental evidence which appeared to establish the existence of a hereditary mechanism of the kind which Hippocrates described has been obtained on a number of occasions. One of the most recent concerns the effect of fertiliser treatment on *Linum usitatissium* (Flax) (Durrant, 1962). Rigorous attention to many points of detail is necessary in order to exclude the possibility of selection, and no claim for the existence of a direct adaptive

influence of the environment on inheritance has gained general acceptance. In view of this lack of confirmation, it is remarkable that Hippocrates' hypothesis remained unchallenged for 23 centuries. Numerous experiments on the crossing of different species or varieties of plants and animals were performed during the 18th and 19th centuries, but although these studies failed to provide support for the classical theory, they did not lead to any alternative hypothesis. That is, of course, with the notable exception of Mendel's work. Some of the most promising earlier experimental studies were made by Knight and by Goss with *Pisum sativum* (Edible Pea) and since this was the plant which Mendel subsequently used so successfully, their work will be described.

Knight (1799) lived near Ludlow, England, and his primary intention was to obtain new and improved varieties of fruits and vegetables. He chose the Edible Pea as his initial experimental material, because of its short generation time, the numerous varieties available and the self-fertilizing habit, which made the protection of the flowers from insects carrying pollen unnecessary.

He crossed two varieties which differed in colour. One was unpigmented, having green stems, white flowers and colourless (white) seed-coats, while the other had purple stems and flowers and grey seed-coats. He found that when an unpigmented plant was pollinated by a pigmented one, only pigmented progeny were obtained the next year (the first filial generation), but these on self-pollination, or on pollination by unpigmented plants, produced some pigmented and some unpigmented plants in the succeeding year. This diversity was manifest even when the peas from within one pod were sown. However, he did not record the numbers of the two kinds, either in the progeny of individual plants, or in total, and so failed to discover the underlying mechanism of heredity and was able merely to deduce that there was a 'stronger tendency' to produce coloured than colourless plants.

Knight also established that reciprocal crosses gave identical results. Thus a tall variety pollinated by a dwarf one, and *vice versa*, gave plants which were the same as regards their vigour of growth, the size of the seeds and the season of maturity. Unknown to Knight, the identity of the progeny from reciprocal crosses had already been discovered by Kölreuter (1763) using species of *Nicotiana* and other flowering plants.

Goss (1824) made similar discoveries to those of Knight, but took the analysis a little further. He lived at Hatherleigh in Devonshire, and had also been attempting to produce new varieties of vegetables. He had removed the stamens from a normally green (blue) seeded pea, pollinated it from a yellow (white) seeded variety, and had been surprised to find that the seeds set (the first filial generation) were yellow like the male parent (Fig. 1.1). He sowed these the following year and was equally surprised when, after self-pollination, he found some pods with all green, some with all yellow, and many with both green and yellow peas in the same pod (the second filial generation) (Fig. 1.2). On sowing these the next year, he found on further 'selfing' that whereas the green peas bred true and gave only green progeny, the yellow yielded some pods with all yellow and some with both green and

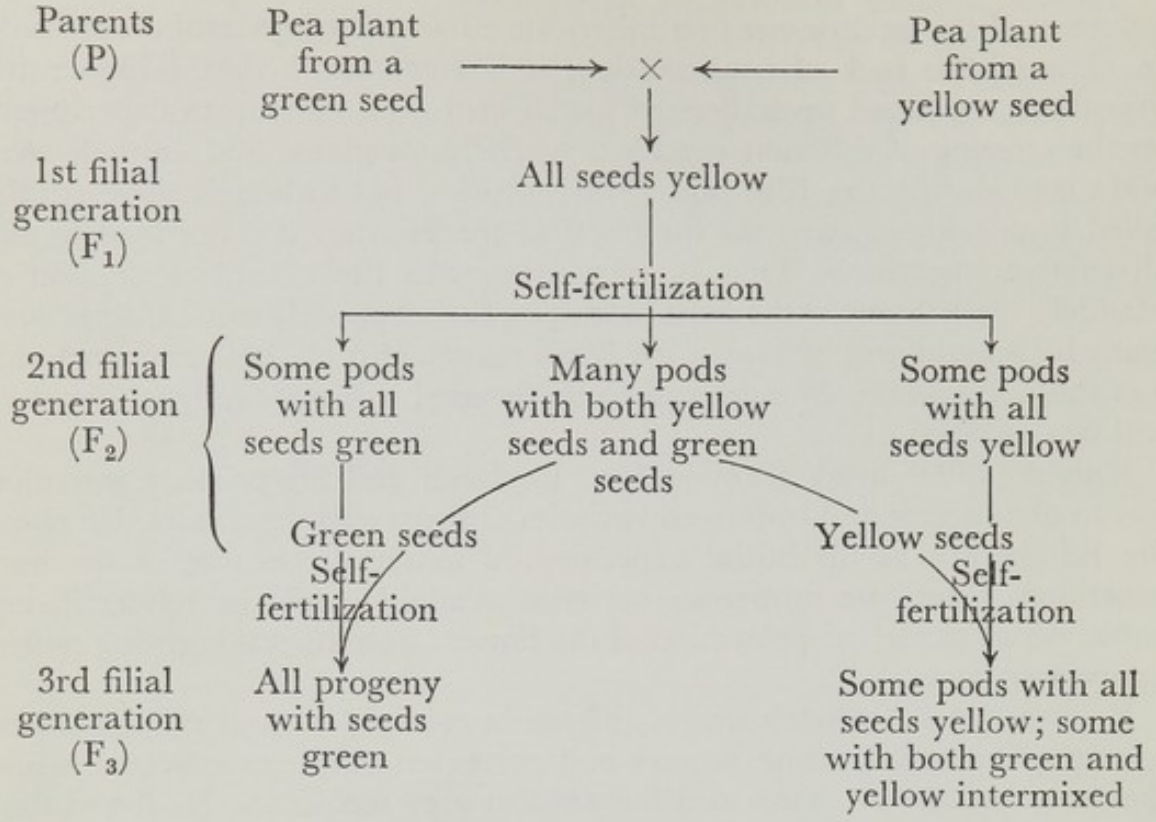


FIGURE 1.1 Goss' results from crossing green and yellow peas (*Pisum sativum*).

yellow peas intermixed (the third filial generation) (Fig. 1.1). Forty-two years later, Mendel (1866) reported similar results. However, Goss, like Knight, did not count the numbers of the two kinds of peas (or if he did count them, he failed to see the significance of the figures and did not publish them), and so he failed to discover the hereditary mechanism which Mendel found.

The studies by Knight and by Goss with peas illustrate the kind of result that was obtained in the 18th and 19th centuries with many organisms. The experimental work at this time appears to have been designed, either to obtain new and improved varieties of animals and plants for use in agriculture or

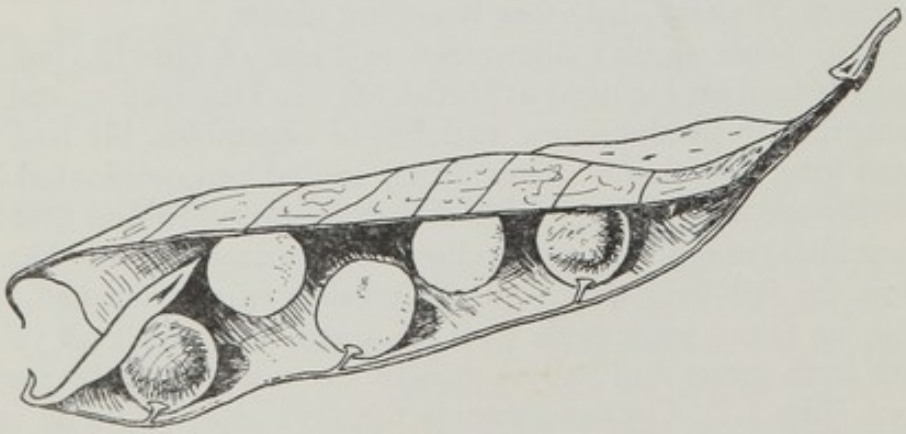


FIGURE 1.2 Drawing of a pea pod with seeds of two colours, similar to that used to illustrate Goss' paper.

horticulture, or with the object of understanding the nature of species by means of artificial hybridizations, rather than with the specific intention of obtaining information about the mechanism of heredity. Probably this is the reason that the experimental results were not recorded with sufficient detail and precision for anything about heredity to emerge except 'an inscrutable medley of contradictions' (Bateson 1909, page 2). To take one example, Goss states that his second generation yellow peas produced, on selfing, some pods with a mixture of green peas and yellow peas, and some with all the peas yellow. If he had studied the plants more carefully he would have found no doubt, as Mendel did, that one can substitute 'plants' for 'pods' in the previous sentence; in other words some of the second generation yellow peas breed true. He would then have been much nearer to understanding the underlying mechanism of inheritance.

2. *The theory of indirect inheritance through particulate determinants*

§ 2.1 Mendel's experimental results

Mendel (1866) had made crossing experiments with *Pisum sativum* similar to those of Knight, Goss and others but, unlike them, he recorded the numbers of progeny of each kind. This was the rewarding advance which enabled him, by a stroke of genius, to detect the underlying mechanism and so to put forward an entirely new hypothesis concerning heredity.

One of the character-differences which Mendel studied was the same as Knight had used about 60 years earlier, namely the presence or absence of pigmentation in the plant (stems, flowers and seed-coats). Like Knight, Mendel found that the progeny from a cross between the two forms were pigmented (the first filial generation), and that, on self-pollination of these, both pigmented and unpigmented plants were obtained (the second filial generation). But Mendel went further and counted the numbers of each kind. He found that among 929 plants, 705 were pigmented and 224 were not, and he observed that these frequencies closely approximate to $\frac{3}{4}$ and $\frac{1}{4}$ of the total (696·75 and 232·25), respectively. The character—presence of pigment, in this instance—which is manifest in all the immediate progeny of the cross, and in $\frac{3}{4}$ of the following generation, Mendel called the *dominant* character, the other being the *recessive*. He also confirmed Kölreuter's and Knight's observations that reciprocal crosses (*A* female $\times$ *B* male, and *B* female $\times$ *A* male) give similar results.

Mendel also worked with the character-difference which Goss (1824) had used, confirming that yellow seed (embryo) is dominant to green, and that both kinds appear in the second generation after crossing. Unlike Goss, Mendel counted his peas, and recorded that out of 8023, 6022 were Yellow* and 2001 green, or again a very close approximation to $\frac{3}{4}$ (= 6017·25) and $\frac{1}{4}$ (= 2005·75). Mendel confirmed Goss' third-generation observations, namely, that the green peas bred true, whereas the Yellow often did not. But Mendel went much further than this. Of 519 such Yellow seeds, he found that 166 bred true, while 353 did not (but gave Yellow and green seeds in the proportion of 3 to 1 like the previous generation). Mendel observed that the frequencies 166 and 353 are a close fit with $\frac{1}{3}$ (= 173) and

* A capital initial letter is used for dominant characters, lower case for recessive ones.

$\frac{3}{4}$ (= 346) of the total, respectively. This implied that the second-generation ratio of 3 dominants to 1 recessive was really a ratio of 1 pure-breeding dominant to 2 impure dominant to 1 recessive (always pure-breeding). Mendel pursued his inheritance studies through 5 or 6 generations and showed that the pure-breeding types in the second filial generation remained pure-breeding, and the others gave a 1:2:1 ratio in each generation.

Essentially similar results to those just described were obtained by Mendel for a total of 7 character-differences in *P. sativum*. In the first generation from a cross, one character was always dominant to its alternative, and in the second generation showed a 3 to 1 ratio, and this, on further analysis, was shown to be a disguised 1 to 2 to 1 ratio. What was the significance of these simple numerical ratios?

§ 2.2 Mendel's theory

In building an hypothesis to account for his results, Mendel introduced symbols, *A* for the dominant and *a* for the recessive character. This use of

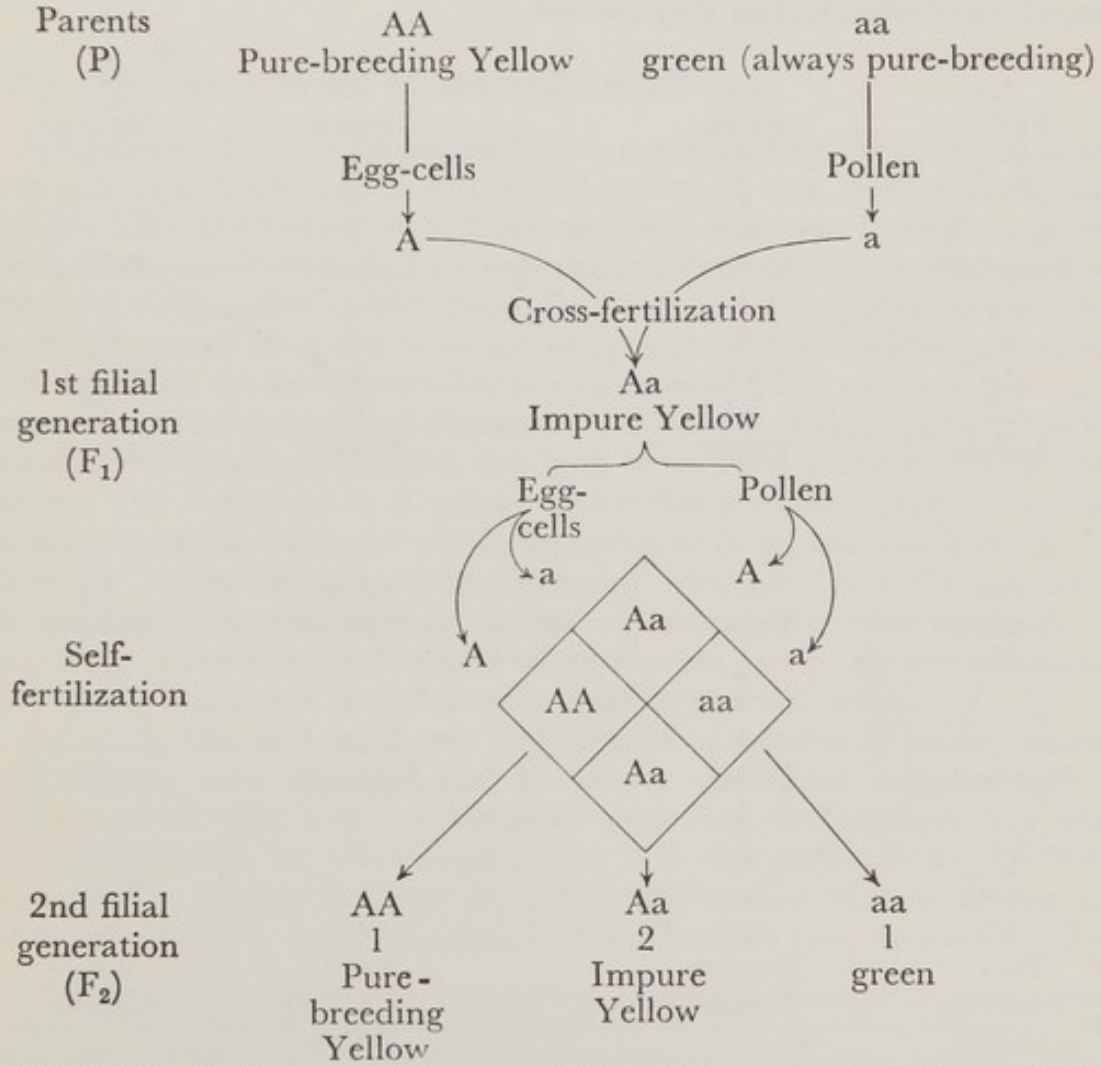


FIGURE 2.1 Representation of one of Mendel's experiments, using symbols (*A* and *a*) for the hypothetical hereditary determinants (*A* for Yellow, *a* for green seed-colour).

symbols is most significant, as it implies the existence of something which 'stands for' the character. Although Mendel used 'Charakter' or 'Merkmal' almost throughout his paper, it is clear that he was thinking in terms of 'factors' or 'determinants' responsible for the manifestation of the character. This is the crucial feature of his theory of heredity, that characters are not 'transmitted' directly from generation to generation as the classical theory supposed, but that there exist discrete particles responsible for the appearance of particular characters. Furthermore, each individual receives one particle from each of its two parents in respect of a particular character-difference; and these particles do not influence one another in any way but separate uncontaminated at the time of formation of the reproductive cells. If *A* denotes the particle which determines Yellow seeds and *a* that for green seeds, then on this hypothesis, Mendel's experiment described above can be represented as in Fig. 2.1. A further corollary implicit in the hypothesis was that egg-cells carrying *A* were equally likely to be fertilized by pollen cells carrying *A* or those carrying *a*, and similarly with egg-cells carrying *a*. In other words, fertilization was at random between egg-cells and pollen-cells, irrespective of what factors they carried.

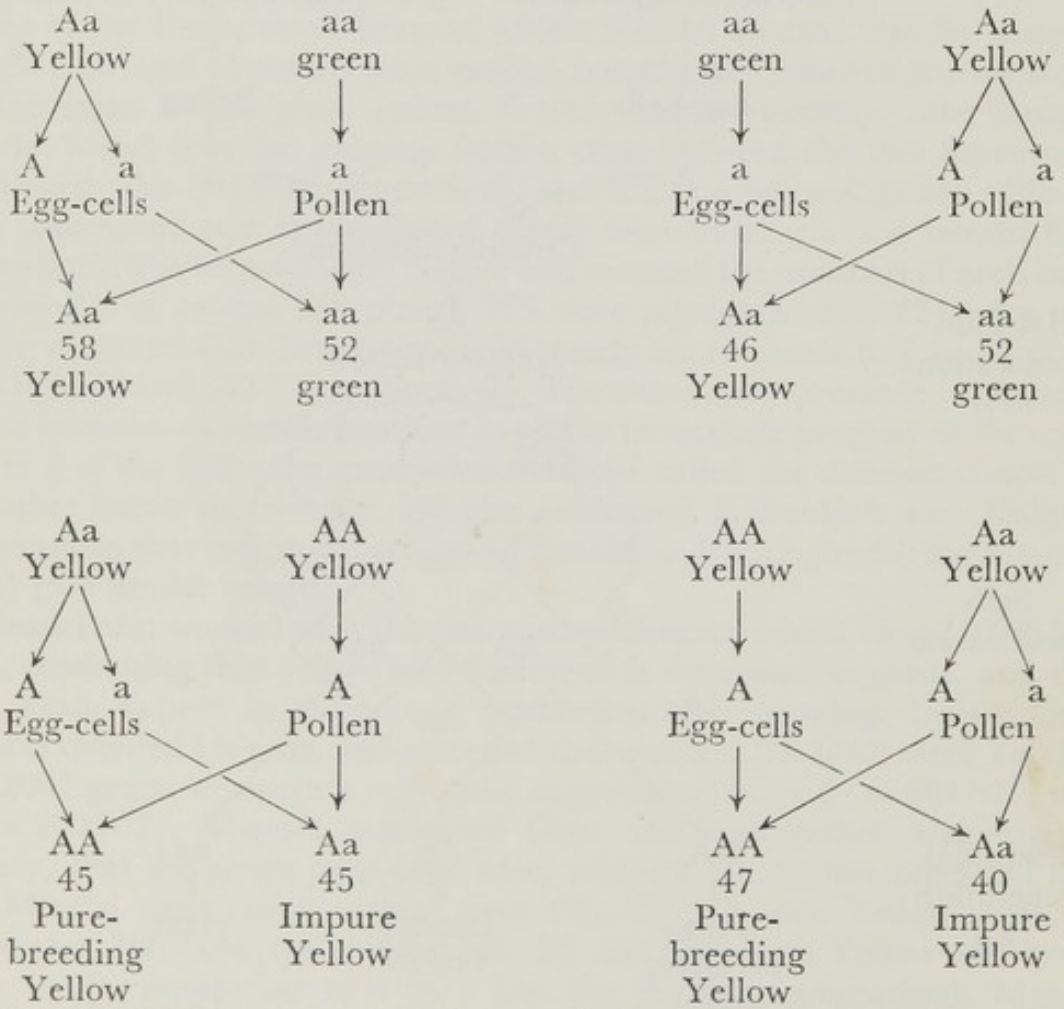


FIGURE 2.2 Representation of a set of back-cross experiments used by Mendel to test the validity of his theory of heredity. *A* = determinant for Yellow seed-colour, *a* for green.

In order to test the validity of this theory of heredity, Mendel devised a series of experiments. The Yellow peas obtained in the first generation from a cross between pure-breeding Yellow and green (necessarily pure-breeding) were germinated and the flowers on the resulting plants, instead of being allowed to self-pollinate, were crossed with the parental forms. On Mendel's hypothesis, from such a 'back-cross' to the green parent, a 1 to 1 ratio of Yellow to green peas is expected and this prediction was confirmed experimentally: the first generation Yellows as female parent crossed with green as male parent gave 58 Yellow and 52 green peas as progeny, and the reciprocal cross (green as female parent, first generation Yellow as male parent) gave 46 Yellow and 52 green. Comparable results were obtained on back-crossing the first generation Yellow peas to the Yellow parental type, but here it was necessary to grow the progeny and allow them to self-pollinate in order to reveal the 1:1 ratio of pure-breeding and impure Yellows. These experiments confirming the hypothesis are shown diagrammatically in Fig. 2.2. Mendel made similar tests with all the 7 character-differences in peas with which he worked, and in every case he obtained the expected 1:1 ratio.

§ 2.3 The rediscovery of Mendel's work

Mendel's work made no impression on contemporary thought and indeed was almost entirely overlooked for 34 years. It was 'rediscovered' independently by De Vries (1900), Correns (1900) and von Tschermak (1900). Each of these authors had made experiments similar to those of Mendel and obtained comparable results. Thus, De Vries had found that various character-differences in a whole range of species of flowering plants showed dominance in the first generation from a cross and 3:1 ratios in the second generation. The species and characters are listed in Table 2.1. Correns had worked with *Zea mays* (Maize) and both he and von Tschermak with *Pisum sativum*. The characters in *P. sativum* which they studied included those used by Knight and by Goss and which, unknown to them, had also been used by Mendel. Correns introduced the expression 'Mendels Regel' (Mendel's law) for the basic principle, namely, the separation or segregation at the time of formation of the reproductive cells of the discrete particulate determinants of alternative characters, and their reassociation at fertilization.

So, when Mendel's work was rediscovered, a number of his experiments had already been repeated and his results confirmed independently by two observers. The truth of Mendel's work was thus immediately established. Moreover, its wide applicability was demonstrated by De Vries' experiments. Further evidence for this came from the work of Bateson and Saunders (1902)*, who established that Mendel's law applied to *Gallus*

* Bateson and Saunders introduced several terms which have since been generally adopted: *allelomorph* (now often abbreviated as *allele*) for each of the alternative factors responsible for a particular character-difference; *heterozygote* for the product of fertilization of gametes differing by an allelomorph; and *homozygote* for the product of fertilization of gametes carrying the same allelomorph. They also proposed the symbols P for the parental generation, F_1 for the first filial generation, F_2 for the second filial generation, and so on.

TABLE 2.1 Character-differences found by De Vries (1900) to show Mendelian inheritance as indicated by 3:1 ratios in the second filial generation.

Name of Plant	Characters	
	Dominant	Recessive
Agrostemma githago (Corn Cockle)	Normal reddish-purple petals	Petals pale-coloured
Aster tripolium (Sea Aster)	Normal blue flower-colour	White flower-colour
Chelidonium majus (Greater Celandine)	Normal pinnately cut leaves and petals	Laciniate leaves and petals
Chrysanthemum roxburghi	Normal yellow flower-colour	White flower-colour
Coreopsis tinctoria	Normal yellow flower-colour	Brown flower-colour
Datura tatula and D. stramonium (Thorn Apple)	Purple stem- and flower-colour	Green stem- and white flower-colour
——	Thorny fruit	Unarmed fruit
Hyoscyamus niger (Henbane)	Normal purple-veined corolla	Corolla pale-coloured
Oenothera lamarckiana De Vries (Evening Primrose)	Normal style	Short style
Papaver somniferum (Opium Poppy)	Dark spot at base of petals	Petals without basal dark spot
——	Normal single flower	Double flower
Silene alba (White Campion)	Normal hairy shoots	Glabrous shoots
Silene dioica (Red Campion) and *S. alba*	Red petal colour	White petal colour
Solanum nigrum (Black Nightshade)	Normal black fruit	Green fruit
Trifolium pratense (Red Clover)	Normal red flowers	White flowers
——	Quinquefoliate leaves	Normal trifoliate leaves
Veronica longifolia	Normal blue flower-colour	White flower-colour
Viola cornuta	Normal blue flower-colour	White flower-colour
Zea mays (Maize)	Normal starchy endosperm	Sugary endosperm

domesticus (Domestic Fowl), as well as to further plants. In *G. domesticus* they found an extra toe was dominant to the normal foot; pea comb was dominant to single comb; rose comb was also dominant to single comb; and white shanks and bill were dominant to yellow. Each of these character-differences appeared in a 3:1 ratio of dominants to recessives in F_2. Simultaneously and independently, Cuénot (1902) also established that Mendelian inheritance occurred in animals, when he showed that the normal grey coat-colour in *Mus musculus* (House Mouse) was dominant to albino in F_1 from a cross, and segregated to give 198 grey and 72 albino in F_2.

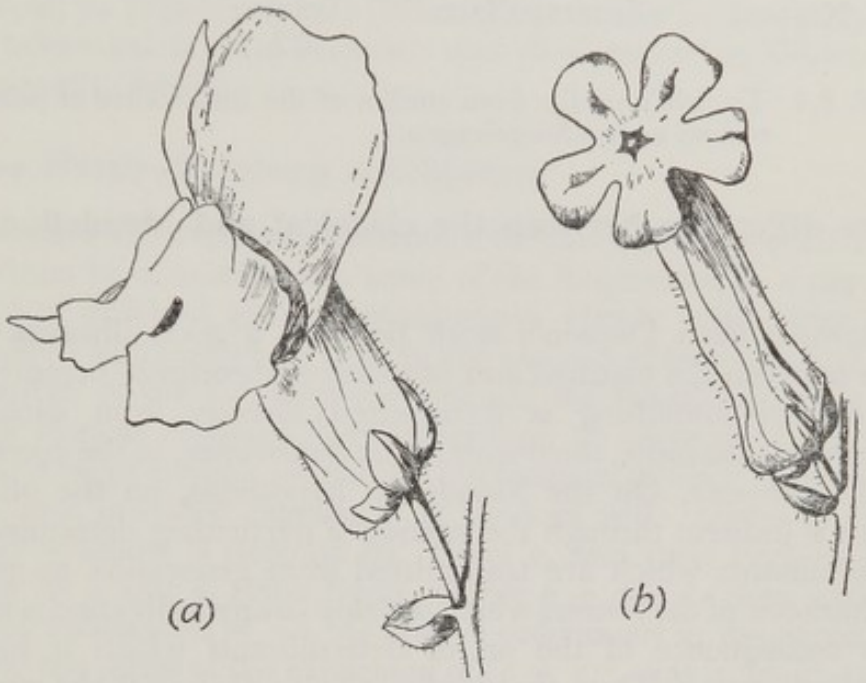


FIGURE 2.3 Drawings of (*a*) normal, and (*b*) peloric *Antirrhinum majus* (Snapdragon).

Bateson and Saunders (1902) drew attention to the fact that many studies on inheritance dating from before 1900, previously inconclusive, were now explicable in terms of Mendelian inheritance. They quoted, for instance, the work of Darwin (1868, vol. 2, Chapter 14) with peloric *Antirrhinum majus* (Snapdragon). This form differs from the normal in having a regular radially-symmetrical flower instead of the normal irregular two-lipped structure (Fig. 2.3). Darwin's results are shown in Fig. 2.4. They were interpreted by him as indicating that the tendency to produce normal flowers prevailed in the first filial generation from the cross between the two forms, whilst 'the tendency to pelorism appeared to gain strength by the intermission of a generation', and prevailed to a large extent in the second filial generation. But on Mendel's theory of heredity it is merely necessary to postulate that the factor for pelorism is recessive to the normal. Darwin's F_2 frequencies, omitting the two intermediate plants, are in good agreement with the 3:1 ratio expected on the Mendelian hypothesis. Later studies have confirmed the Mendelian explanation.

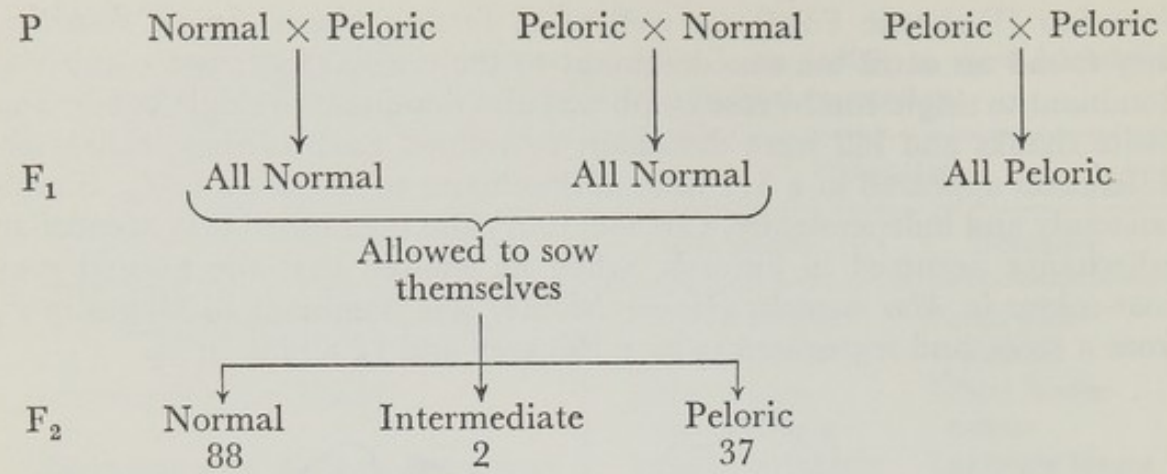


FIGURE 2.4 Darwin's results from studies of the inheritance of peloric *Antirrhinum majus* (Snapdragon).

§ 2.4 The difference between the classical and Mendelian theories

The example from Darwin's work provides a good illustration of the difference between the classical and Mendelian theories of heredity. On the classical theory, something is transmitted directly from each part of the organism, for example, the flower of the *Antirrhinum*, to the corresponding part in the progeny. On the Mendelian hypothesis, on the other hand, inheritance is indirect through the agency of particulate determinants. It is these determinants which are transmitted from generation to generation, and the character of the flowers which develop in any individual is merely the fortuitous consequence of the set of determinants which it happens to receive at fertilization. Thus, the classical theory deals with the tendencies of characters to gain strength or lose strength, while Mendel's is concerned with chance combinations in pairs of dominant and recessive particulate determinants of characters. The fundamental difference is that on the classical theory inheritance is direct and hence the extent of transmission of a particular character to the progeny may be influenced by the character itself, whereas on the Mendelian theory the determinant of a character is considered to be in no way modified by its presence in an organism, whether it possesses that character or not, and hence the inheritance of a character is thought to be unaffected by the character itself. Thus, on the Mendelian theory, the inheritance of mutilations, such as Hippocrates quoted in support of the classical theory, is impossible.

§ 2.5 The diversity of Mendelian characters

Bateson, Saunders and Punnett (1905) established one of the first instances in Mendelian inheritance of the occurrence of incomplete dominance, that is, where the heterozygote is intermediate between the two homozygotes. They found that the so-called 'blue' Andalusian variety of *Gallus domesticus* (really a grey) is the heterozygote from a cross between a black and a white

variety. Many further instances of incomplete dominance were established soon afterwards, both in animals and in plants. Dominance and recessiveness were thus seen not to be an essential part of Mendel's theory of heredity, the central notion of which is the coming together at fertilization, and the separation at gamete-formation, of pairs of particulate determinants.

Within a few years of the rediscovery of Mendel's work, innumerable examples of Mendelian inheritance had been established. Bateson (1909), without any attempt at completeness, listed about 100 examples in plants and a similar number in animals. Both the organisms and the characters involved were highly diverse, including susceptibility to *Puccinia glumarum* (Yellow Rust) in *Triticum aestivum* (Wheat); eye-colour in *Homo sapiens* (Man); waltzing behaviour in *Mus musculus*; and shell colouring in *Helix hortensis* and *H. nemoralis* (Snails).

§ 2.6 The Hardy-Weinberg equilibrium

Confirmation of Mendelian inheritance for particular character-differences can sometimes be obtained from study of the frequencies in a population of individuals of different genetic constitutions. Hardy (1908) and Weinberg (1908) showed independently that, in the absence of mutation* or selection, the frequencies of the two homozygotes and the heterozygote for a pair of alleles will remain constant from generation to generation, provided the population is large enough for chance fluctuations in their proportions to be neglected, and provided crossing is at random with respect to the alleles. If the pair of alleles is denoted by A and a, and their frequencies in the population by p and q, respectively, where $p + q = 1$, then with random crossing the frequencies of AA, Aa and aa individuals in the population will be given by the terms of the expansion of $(p + q)^2$, that is, by p^2, $2pq$ and q^2, respectively. The frequencies of all the possible crosses, such as $AA \times Aa$, $Aa \times Aa$, and so on, can then be expressed in terms of p and q, and likewise with their progeny. When the frequencies of AA, Aa and aa progeny from the various crosses are summed, it is found that they are given by the expressions p^2, $2pq$ and q^2, respectively. In other words, the population is in equilibrium, and the relative proportions of individuals with the different genetic constitutions are the same as in the previous generation.

Snyder (1931) discovered that ability to taste phenyl-thiocarbamide was due to a single dominant Mendelian gene, and that in 440 individuals from 100 American families, 301, or 68·5%, could taste it and 139, or 31·5%, could not. There were 9 families in which both parents were taste-deficient, and their 17 children were all taste-deficient also, as expected if this is a recessive character. Assuming that the Hardy-Weinberg equilibrium conditions apply (that is, large population size, random mating with respect to tasting, no differential viabilities or fertilities of the heterozygote or homozygotes, and negligibly low mutation rates), the frequency of non-tasters (q^2) is 0·315. It follows that $q = 0{\cdot}561$, $p = 1 - q = 0{\cdot}439$, $p^2 = 0{\cdot}192$ and $2pq = 0{\cdot}493$; that is, 19·2% of the total population are expected to be

* Mutation is an abrupt change from one allele to another: see § 9.2.

homozygous and 49·3% heterozygous for the dominant gene for tasting ability. This means that $\frac{0{\cdot}192}{0{\cdot}685}$ or 28% of the tasters are homozygous and 72% heterozygous. It is evident that in 72% of families where one parent is a taster and the other not, there will be equal numbers of tasters and non-tasters among the children, while in the other 28% of such families, the children will be expected all to be tasters. Snyder tested 117 children from 51 such families and found that 37 were taste-deficient. The expected number is half of 72% of 117 = 42·1, in agreement with the observed number. In 40 families where both parents were tasters, he found 16 taste-deficient children out of a total of 106. The expected number is $\frac{1}{4} \times \left(\frac{72}{100}\right)^2 \times 106 = 13{\cdot}7$. Thus, the numbers of tasters and non-tasters among the children in both these classes of families are in agreement with the Hardy-Weinberg predictions. This confirms that the taste deficiency is due to one recessive gene, and this confirmation has been obtained without the need to test tasting ability over several generations.

One of the conditions for Hardy-Weinberg equilibrium is that there shall be no selection favouring one or other allele. In practice, the maintenance of a pair of alleles in a population, if not due to recurrent mutation, is likely to be due to *balanced polymorphism*, that is, the possession by each allele of certain advantages over the other, depending on the conditions. It is a simple possibility if the heterozygote has an advantage over either homozygote. An example of this is provided by heterozygotes for a pair of alleles, *A* and *S*, affecting human haemoglobin. Allele *A* gives normal adult haemoglobin, and *S* gives sickle-cell haemoglobin (see Chapter 13). Allison (1954) showed that the heterozygote, *AS*, has increased resistance to malaria compared with normal *AA* individuals. On the other hand, sickling homozygotes, *SS*, suffer from a severe anaemia which is usually lethal in childhood. As expected, the *S* allele occurs in human populations only where malaria is endemic, such as in West Africa, or in populations recently derived from such areas.

§ 2.7 Non-Mendelian inheritance

Although Mendelian inheritance appears to be so widespread, it is not universal. One of the first exceptions to be established was through the work of Correns (1909). He had been studying the inheritance of a number of pale-leaved or variegated forms in various flowering plants. A strain of *Urtica pilulifera* (Roman Nettle) with yellowish-green leaves, and a variegated strain of *Lunaria annua* (Honesty) with white-margined leaves were found to be inherited as Mendelian recessives. In *Mirabilis jalapa* (Marvel of Peru), a strain called *chlorina* with leaves wholly yellowish-green, another called *variegata* with leaves variegated with yellowish-green, and a third called *striata* with striped flowers, were all found to show Mendelian inheritance. However, a fourth strain of this plant, called *albomaculata*, with leaves variegated with yellowish-white, was found to show a different kind of

inheritance. Correns observed that, in addition to the variegated ones, occasional shoots on the *albomaculata* plants were normal, being wholly green, while others were wholly white. He found that flowers on green shoots gave only green progeny, irrespective of whether the pollen came from green, white or variegated shoots. Similarly, whatever the source of pollen, flowers on white shoots gave only white progeny, which necessarily died soon after seed-germination owing to the lack of chlorophyll for photosynthesis. Flowers on variegated shoots, again irrespective of the type of shoot from which the pollen was obtained, gave three kinds of progeny: wholly green; wholly white; and variegated; and moreover, the proportions of the three kinds were highly variable, never consistently fitting any particular ratio. Plants or branches with only traces of white gave a higher proportion of wholly green progeny than did strongly variegated individuals or shoots.

Here were results differing markedly from those expected on Mendel's hypothesis, notably in the maternal inheritance, with divergent results from reciprocal crosses, and in the lack of constant proportions of the different kinds in segregating progenies. Hypotheses which have been put forward to explain these results will be discussed in Chapter 5, when other examples of non-Mendelian inheritance will be described. However, it has been evident ever since this work by Correns that Mendelian inheritance, although widespread and abundant, is not the only method of inheritance in living organisms. The relative importance of the different methods of inheritance is discussed in Chapter 18.

3. *The theory of independent inheritance of determinants for different characters*

§ 3.1 Mendel's results for two character-differences

Mendel (1866) in his classic paper on inheritance in *Pisum sativum* did not confine himself merely to the study of the inheritance of various character-differences considered in isolation. He made experiments in which the parents differed in two or three characters. Thus, a pure-breeding strain with round (that is, smooth) yellow seeds was crossed with a pure-breeding strain with wrinkled green seeds. The F_1 seeds were all Round Yellow, these being the dominant characters. In F_2 he obtained 556 seeds, which comprised 315 Round Yellow, 101 wrinkled Yellow, 108 Round green and 32 wrinkled green. These figures are in good agreement with a ratio of 9:3:3:1. Similar results were obtained with all the combinations of character-differences in peas which Mendel chose to test, apart from such features as presence or absence of pigment in the seed-coat and flower, where Mendel confirmed Knight's observation that these are merely two aspects of one character-difference: the presence or absence of pigment in the plant as a whole.

§ 3.2 Mendel's hypothesis

As an hypothesis to account for his results, Mendel postulated that when two character-differences are present each is inherited independently. The 9:3:3:1 ratio is then predicted, for it is the consequence of superimposing two 3:1 ratios, one for each character-difference. Its derivation is shown in Table 3.1, where R represents the determinant for Round seeds, r for wrinkled, Y for Yellow and y for green. Mendel assumed that at pollen formation in the F_1 plants, pollen-grains with the four possible combinations of the two pairs of factors, that is, RY, Ry, rY, ry, arise with equal frequency, and similarly at egg-cell formation. Mendel also assumed that fertilization is at random between the different kinds of pollen and egg-cells. It then follows, as indicated in Table 3.1, that on the average $\frac{9}{16}$ of the combinations will carry both R and Y, $\frac{3}{16}$ will carry R but not Y, $\frac{3}{16}$ Y but not R, and $\frac{1}{16}$ neither R nor Y.

TABLE 3.1 The proportions of progeny of different kinds expected in F_2 from a cross between pure-breeding strains differing in two characters, on Mendel's hypothesis that in the double heterozygote the four possible combinations of two pairs of factors arise with equal frequency, both in the formation of the pollen and of the egg-cells, and that fertilization is at random. R = factor for Round, r for wrinkled, Y for Yellow, y for green seeds in *Pisum sativum*. (*a*) Individual frequencies. (*b*) Totals.

(*a*)

Reproducive Cells from F_1 Heterozygote			Pollen			
			RY 1	*Ry* 1	*ry* 1	*rY* 1
Egg-cells	*RY*	1	*RRYY* 1	*RRYy* 1	*RrYy* 1	*RrYY* 1
	Ry	1	*RRYy* 1	*RRyy* 1	*Rryy* 1	*RrYy* 1
	ry	1	*RrYy* 1	*Rryy* 1	*rryy* 1	*rrYy* 1
	rY	1	*RrYY* 1	*RrYy* 1	*rrYy* 1	*rrYY* 1

(*b*)

Totals	*RY* $\frac{9}{16}$
	Ry $\frac{3}{16}$
	ry $\frac{1}{16}$
	rY $\frac{3}{16}$

Mendel confirmed this hypothesis in two ways. Firstly, he bred from his F_2 plants, and showed that the Round Yellow, Round green and wrinkled Yellow peas consisted of the appropriate mixture of pure-breeding and impure types and moreover in the appropriate proportions, while the wrinkled green peas bred true. Secondly he backcrossed the F_1 plants to the parental forms, and demonstrated that such crosses gave a 1:1:1:1 ratio in the progeny. Thus Round Yellow F_1 crossed with wrinkled green gave a progeny of 55 Round Yellow, 51 Round green, 49 wrinkled Yellow, and 53 wrinkled green seeds.

When Mendel's work was rediscovered, confirmation of the independence of inheritance of different character-differences was immediately available. Thus, De Vries (1900) reported an approximation to a 9:3:3:1 ratio in F_2 from a cross between a white trifoliate and a red quinquefoliate strain of *Trifolium pratense* (Red Clover), the latter two characters being dominant; and von Tschermak had unknowingly confirmed Mendel's results for Round Yellow and wrinkled green Peas. Bateson and Saunders (1902) established 9:3:3:1 ratios in F_2 in respect of flower-colour and fruit prickliness in *Datura* (Thorn Apple), and in respect of type of comb and number of toes, and other pairs of character-differences, in *Gallus domesticus*.

§ 3.3 Bateson, Saunders and Punnett's discovery of partial linkage

Bateson, Saunders and Punnett (1905), working with *Lathyrus odoratus* (Sweet Pea) were the first to discover an exception to the law of independence. In an F_2 progeny 381 plants with coloured petals comprised 305 with a purple pigment and 76 with a red pigment. These figures are a rather poor fit with a 3:1 ratio, but later work established this ratio, indicating that purple differs from red by a single dominant factor. The plants also showed diversity in respect of another character-difference, namely, shape of pollen-grain, whether elongated (long) or disc-shaped (round). This character-difference had been chosen for study in the hope (unavailing as it transpired) that it might reveal more precisely when segregation of character-differences took place, for Mendel had postulated its occurrence at the time of formation of the pollen and egg-cells. However, all the pollen-grains on any individual plant were found always to be alike.* By a coincidence, the 381 plants showed the same proportion with the two different shapes of pollen (305 long, 76 round) as for the two different flower colours. Although the fit with a 3:1 ratio is poor, later work confirmed its truth, and hence established that long-pollened plants differ from round-pollened by a single dominant factor.

When the 381 plants were classified simultaneously for pigment colour and pollen shape, a startling discovery was made. The relative frequencies of the four classes were quite different from those expected on the basis of all

* Pollen characters do not always behave like this—see § 5.4.

previous work. The results obtained were:

284 (74·6%) with Purple flowers and Long pollen
21 (5·5%) with Purple flowers and round pollen
21 (5·5%) with red flowers and Long pollen
and 55 (14·4%) with red flowers and round pollen.

More precise frequencies, based on 6952 F_2 plants derived from the same cross (Purple Long × red round) gave:

4831 (69·5%) Purple Long
390 (5·6%) Purple round
393 (5·6%) red Long
and 1338 (19·3%) red round (Punnett, 1917).

This *coupling* of 'Purple' with 'Long' (the two dominant characters), as Bateson, Saunders and Punnett called it, implies that at the time of pollen

TABLE 3.2 The proportions of progeny of different kinds expected in F_2 from a cross between pure-breeding strains of *Lathyrus odoratus* with purple flowers and long pollen and with red flowers and round pollen on the assumption that reproductive cells of constitution *PL* and *pl* each have a frequency of 44%, and *Pl* and *pL* of 6%, and with fertilization at random. *P* = factor for Purple, *p* for red flowers; *L* for Long, *l* for round pollen. (*a*) Individual frequencies. (*b*) Totals.

(*a*)

Reproductive Cells from F_1 Heterozygote			Pollen			
			PL 4·4	*Pl* 0·6	*pl* 4·4	*pL* 0·6
Egg-cells	*PL*	4·4	19·36%	2·64%	19·36%	2·64%
	Pl	0·6	2·64%	0·36%	2·64%	0·36%
	pl	4·4	19·36%	2·64%	19·36%	2·64%
	pL	0·6	2·64%	0·36%	2·64%	0·36%

(*b*)

Totals	*PL* 69·36% *Pl* 5·64% *pl* 19·36% *pL* 5·64%

and egg-cell formation in the F_1 plants, the four different combinations of the factors *P* (Purple) and *p* (red flower-colour), and *L* (long) and *l* (round pollen) do not arise with equal frequency, but that *PL* and *pl* are of appreciably higher frequency than the other two combinations (*Pl* and *pL*). Frequencies of 44% for each of *PL* and *pl*, and of 6% for *Pl* and *pL*, instead of the 25% for each which Mendel would have expected, were found (Haldane, 1919) to give a close fit with the data from the 6952 plants (see Table 3.2).

As Bateson, Saunders and Punnett continued their breeding work with *Lathyrus odoratus*, several other examples of such *partial linkage* between different character-differences were soon found. The cause of partial linkage will be discussed in Chapter 7.

§ 3.4 Doncaster and Raynor's discovery of sex-linkage

Linkage of another kind, in which two of the four possible ways of associating two character-differences appeared not to arise at all, was discovered

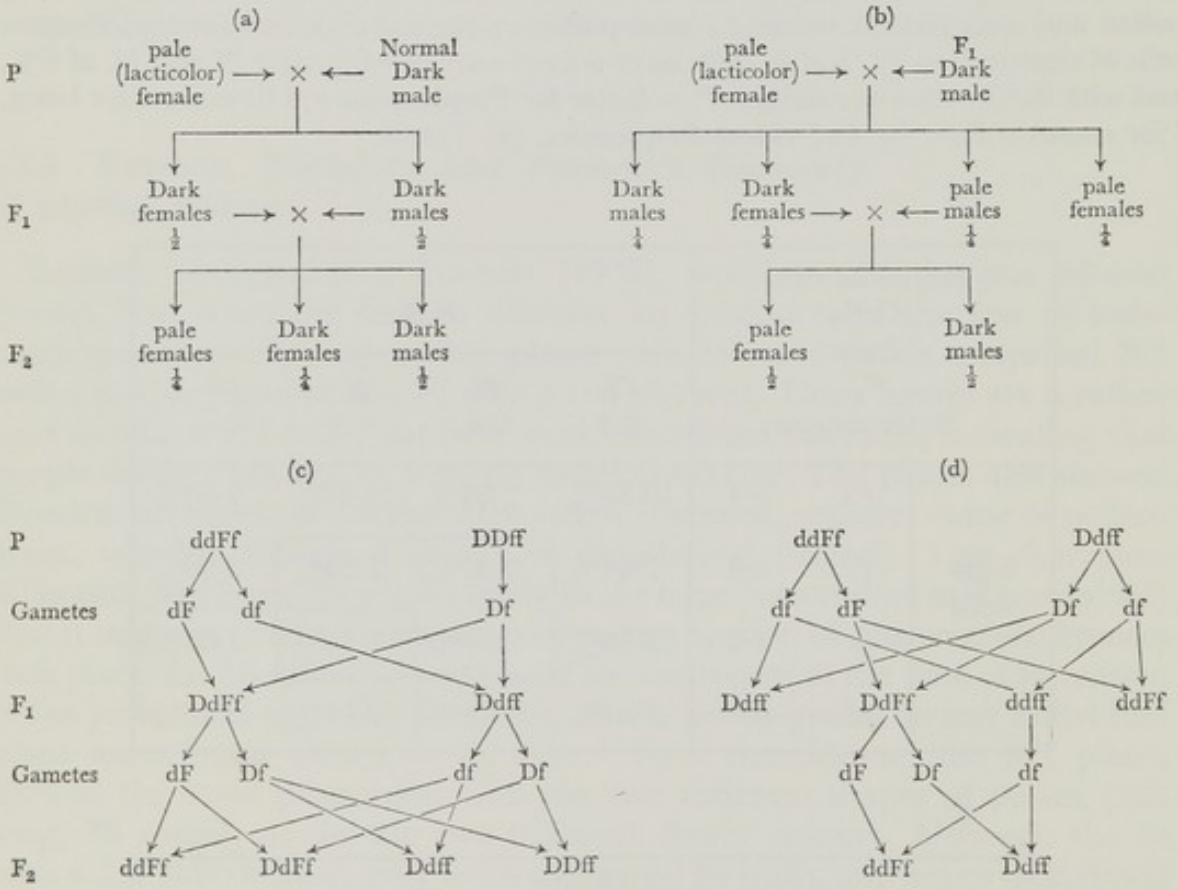


FIGURE 3.1 Doncaster and Raynor's results ((*a*) and (*b*)) from study of the inheritance of the pale (*lacticolor*) form of *Abraxas grossulariata* (Magpie Moth), and Punnett and Bateson's hypothesis ((*c*) and (*d*)) to explain them. *D* = dominant factor for normal Dark form of the moth, *d* = recessive allele for pale form, *F* = dominant factor for Femaleness, *f* = recessive allele for maleness. The hypothesis was later modified: see § 6.11.

by Doncaster and Raynor (1906) working with *Abraxas grossulariata* (Magpie Moth). They studied the inheritance of a pale form of this moth called *lacticolor*, which first appeared in a female individual. They established that the pale character behaved as a normal Mendelian recessive, disappearing in F_1 and reappearing in one quarter of the F_2 and in one half of the backcross progeny, that is, the progeny from crossing the F_1 males (normal) with their mother (pale) (see Fig. 3.1(*a*) and (*b*)). However, the following results were unexpected:

(i) The quarter of the F_2 which were pale were all female, and hence resembled their grandmother (Fig. 3.1(*a*)).
(ii) Pale males from the backcross progeny, when crossed with their normal sisters, gave only pale females and normal males (the grandparental forms), instead of equal numbers of each form in each sex (Fig. 3.1(*b*)).

Earlier work by Cuénot with albino *Mus* and by Bateson and Punnett with comb characters in *Gallus* had always shown character-differences to be equally distributed between the sexes, so this *sex-linkage* in *Abraxas* was unexpected.

For linkage between two pairs of characters (factors *A*/*a* and *B*/*b*) to be detected it is necessary to have an individual heterozygous for both character-differences, in order to provide the four possible kinds of gamete (*AB*, *ab*, *Ab*, *aB*). Hence, to explain Doncaster and Raynor's results, in addition to accepting that the pale form is recessive, Punnett and Bateson (1908) made two postulates:

(1) Sex in *Abraxas* is a Mendelian character, with femaleness dominant to maleness, such that all Females are heterozygous for the sex factors (*Ff*), and all males are the homozygous recessive (*ff*).

The 1:1 ratio of the sexes in each generation was thereby explained, and the normal females, which provide one parent for both the aberrant progenies (i) and (ii) above, would be the double heterozygote, *DdFf*, where *D* is the dominant factor for the normal Dark insect and *d* the recessive factor for paleness.

(2) The double heterozygote (*DdFf*) produces eggs of only two kinds, bearing the factors for Dark male (*Df*) and for pale Female (*dF*), respectively, the other two combinations (*DF* and *df*) not being produced, for some unknown reason.

Punnett and Bateson spoke of this kind of linkage as *repulsion* since the dominant factors stayed apart, and as complete linkage unlike the partial linkage of the *Lathyrus* example discussed earlier. Their hypothesis was found to explain satisfactorily the sex linkage data in Doncaster and Raynor's results (see Fig. 3.1(*c*) and (*d*)).

However, shortly afterwards, Doncaster (1908) made a remarkable discovery. He found that whenever a pale male was crossed with a normal Dark female, all the male offspring were normal and all the females pale.

This was true even when the normal female parent came from a part of the country where the rare pale form had never been seen. This result was totally unexpected as it implied, on Punnett and Bateson's hypothesis, that all female Magpie Moths were heterozygous for the pale character! As will be shown in § 6.11, their hypothesis was later modified so as to make this unlikely deduction unnecessary.

Morgan (1910) reported results with *Drosophila melanogaster* (Fruit Fly) essentially similar to those of Doncaster and Raynor with *Abraxas*, except in one important respect. Morgan studied the inheritance of white eye-colour in *Drosophila* and found this to differ from the normal dull red eyes by a single recessive factor. The white-eyed condition first appeared in a male individual, unlike *Abraxas* where, as already mentioned, the pale form first appeared in a female. This difference was reflected throughout the inheritance studies on the white-eyed condition in *Drosophila*, where the role of the sexes appeared to be exactly reversed compared with *Abraxas*. To explain these results, Morgan put forward an hypothesis identical to that of Punnett and Bateson for *Abraxas*, except that the position of the sexes was reversed, that is, maleness was regarded as dominant to femaleness and the male *Drosophila* was postulated to be heterozygous for the sex factors, instead of the female *Abraxas*. Furthermore, Morgan found that when a white-eyed female *Drosophila* was crossed with a normal Red-eyed male, even if the latter came from an unrelated stock with no known ancestry of white eyes, the progeny always consisted of Red-eyed females and white-eyed males. This was the exact counterpart of Doncaster's discovery with *Abraxas* and implied that all normal red-eyed *Drosophila* males were heterozygous for white eyes! As with *Abraxas*, the hypothesis was subsequently modified to eliminate this unlikely deduction (see § 6.11).

§ 3.5 Conclusion

In spite of a number of exceptions such as those just described, Mendel's hypothesis of independent inheritance of the determinants for different characters was found to hold quite widely, and ultimately was christened 'Mendel's Second Law' by Morgan (1919), the 'First Law' being the fundamental concept of the coming together of the determinants for a particular character-difference at fertilization, and their separating again unchanged at gamete formation.

4. *The theory of Mendelian inheritance for characters showing a continuous range of variation*

§ 4.1 The analysis of quantitative data

The method which Mendel used so successfully to obtain information about the mechanism of heredity was to find a well-marked character-difference in the chosen organism and to cross pure-breeding strains of each kind. The method is clearly dependent on the existence of sharply defined character-differences. However, many characters are not suitable because they do not reveal clear-cut discontinuities. The most familiar

TABLE 4.1 Johannsen's data for the weight-class of 5494 F_2 beans (seeds of *Phaseolus vulgaris*), obtained by self-pollination for two successive generations of 19 beans of diverse origin.

Weight-class in Centigrams	5–15	15–25	25–35	35–45	45–55	55–65	65–75	75–85	85–95
Number of Beans	5	38	370	1676	2255	928	187	33	2

examples of characters which in most organisms commonly show a continuous range of variation are size measurements such as height or weight. In order to study the inheritance of such characters statistical methods are required. Before describing some of the results obtained from study of the inheritance of characters showing a continuous range of variation, it is necessary to consider the techniques for analysing quantitative data. Only a superficial account is given below of a few statistical methods. For a sound treatment of these and other methods, reference should be made to standard works such as Fisher (1963), Bailey (1959) or Snedecor (1956).

Data of Johannsen (1909) for the weight of dwarf bean (*Phaseolus vulgaris*) seeds will be used as an illustration. He weighed 5494 beans and classified them into 9 categories depending on their weight (see Table 4.1). Such data can be plotted as a frequency histogram, as illustrated in Fig. 4.1. Such histograms for biological data commonly approximate to a smooth curve of a

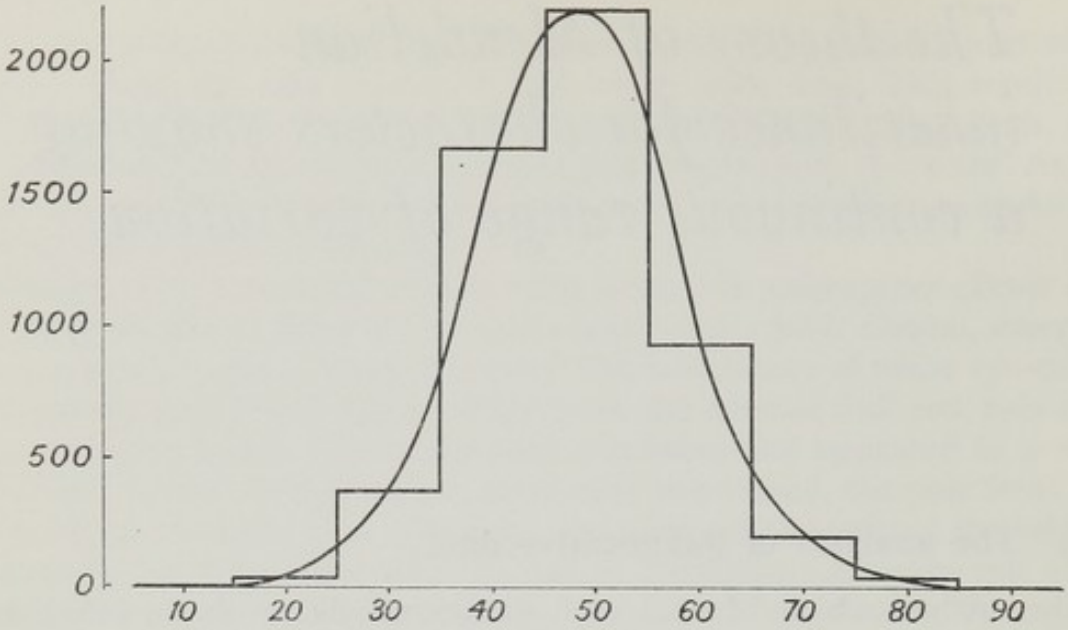


FIGURE 4.1 Frequency histogram for bean weight in *Phaseolus vulgaris* from the data of Johannsen. The number of beans is plotted as ordinate and the weight in centigrams as abscissa. A normal curve has been fitted to the data.

particular kind called the *normal* curve. Non-biological data also frequently fit a normal curve, for example, the frequencies of different numbers of 'heads' (or 'tails') after repeatedly tossing a number of coins simultaneously. With a small number of coins, a stepped graph (histogram) is obtained approximating to the 'binomial distribution' (Fig. 4.2(*a*)), but as the number of coins tossed simultaneously is increased the graph of number of 'heads' (or 'tails') in the throw plotted against the frequency of occurrence of that number approaches the smooth curve of the 'normal distribution' (Fig. 4.2(*b*) and (*c*)). With the coins, whether a 'head' or a 'tail' shall turn up depends on a number of causes which in the long run act with equal frequency in two opposite directions. Similarly, bean weight may be supposed to depend on many factors, such as the position of the seed in the pod, the fertility of the soil and the hereditary constitution of the plant, which again may be supposed on the average to act equally in two directions, some favouring an increased and some a decreased weight.

A set of data which show an approximately normal distribution, such as bean weight in Johannsen's experiment, may be specified by two quantities: firstly, the *mean*, which is the sum of the measurements divided by the total number; and secondly, the *variance* (or its square root, the *standard deviation*) which are measures of the extent to which the various measurements differ from the mean value. The variance is calculated by finding the deviation of each individual measurement from the mean value, squaring each deviation, then summing these squared values, and dividing by the total number of measurements (or strictly speaking, by one less than this total number). On the normal curve, the standard deviation is represented by the horizontal

distance from the position of the mean to the point of steepest slope of the curve on either side. Thus a wide curve, implying considerable variability in the quantity measured, will have a correspondingly large standard deviation. However, the area under the curve measured out as far as the standard deviation on each side is always the same fraction of the total area under the curve, namely 68·26% or approximately $\frac{2}{3}$. This means that $\frac{2}{3}$ of the observations will differ from the mean by less than the standard deviation, and the remaining $\frac{1}{3}$ will exceed it.

A further quantity of use in the analysis of quantitative data is the *covariance*. Suppose pairs of related measurements are available, such as heights of a number of men and of one adult daughter of each of them. Data on human stature usually fit a normal distribution. The covariance of fathers' and daughters' height is obtained by the same method as for variance except that instead of squaring deviations, the deviation of each father's height from the fathers' mean is multiplied by the deviation of the corresponding daughter's height from the daughters' mean. A positive value for the covariance means that on the average when the fathers are tall so are the daughters, and when the fathers are short so are the daughters. A negative value would mean that tall fathers tend to have short daughters, and *vice versa*. If the covariance is zero, a tall father is equally likely to have a tall or short daughter. The co-variance of fathers' and daughters' height divided by the vaiance of the fathers is called the *regression coefficient* of daughters on fathers; if the covariance is divided by the variance of the daughters, this is the *regression coefficient* of fathers on daughters; and if the covariance is divided by the geometric mean (that is, the square root of the product) of the

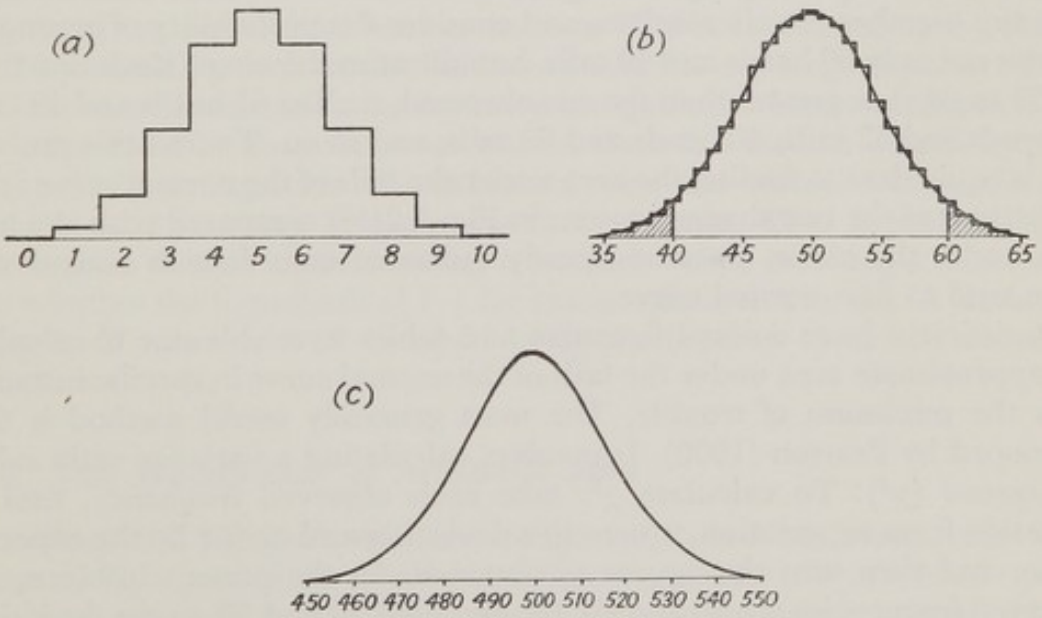


FIGURE 4.2 Frequency histograms or curves for numbers of 'heads' (or 'tails') when (*a*) 10, (*b*) 100, and (*c*) 1000 coins are tossed simultaneously. In (*b*) the shaded areas indicate the probability of obtaining 60 heads and 40 tails or a larger deviation from equality.

two variances (the fathers' and the daughters'), this gives the *correlation coefficient* of the fathers' and daughters' heights. These three coefficients are all measures of the degree of association between the two variables. A value of zero for the correlation coefficient means no association, of +1 means complete positive association, and −1 complete negative association. Intermediate values between −1 and 0 and between 0 and +1 would indicate the appropriate degree of association between the fathers' and daughters' heights.

§ 4.2 The significance of experimental data

The fact that data from the tossing of coins (in sufficient numbers) fit a normal distribution illustrates how the effects of chance can be assessed in testing the significance of experimental data in biology (and elsewhere). Suppose 100 coins are tossed simultaneously. Assuming the coins are not biassed, 50 heads and 50 tails is the most likely result, but there are so many other possibilities such as 49 heads and 51 tails, or 52 heads and 48 tails, that are individually only slightly less likely to occur, that in fact one would be surprised if in a single throw exact equality were achieved. Indeed, on the average 50 heads and 50 tails is expected only about once in every seven or eight throws of 100 coins. The likelihood of each alternative turning up can be precisely calculated. Now suppose at the first toss 60 heads and 40 tails are found. Is this a reasonable result, or should one suspect that the coins are biassed? The probability of this particular ratio turning up by chance is obviously quite low, but this has no significance because, as just indicated, there are so many possible alternative ratios that the probabilities are low for all of them. In order to judge the significance of the 60:40 ratio it is necessary to group together the alternatives, and consider the probability of getting by chance not only 60 heads and 40 tails, but all ratios showing a deviation from 50:50 as great or greater than the one observed, such as 61 heads and 39 tails, 62 heads and 38 tails, 40 heads and 60 tails, and so on. To find this probability is equivalent to finding the area under the tails of the normal curve (that is, the sum of the two shaded regions in Fig. 4.2(*b*)) compared with the total area under the curve, since as already indicated coin data as extensive as these tend to fit a normal curve.

Statisticians have derived formulae and tables to enable one to calculate the approximate area under the tails of the normal curve in specific instances with the minimum of trouble. The most generally useful method is that developed by Pearson (1900). It involves calculating a variance ratio called *khi squared* (χ^2). To calculate χ^2, take each observed frequency, find its deviation from expectation, square this deviation and divide by the expected value, and then sum the figures so obtained. In the present instance, the observed frequencies are 60 and 40, the expected 50 and 50, so the deviations are 10 and 10, and $\frac{(\text{deviation})^2}{\text{expected}}$ is $\frac{100}{50} = 2$ in each instance, and $\chi^2 = 4$. One must then refer to a table showing the values of χ^2 associated with particular areas under the tails of the normal curve. From the way in which

χ^2 is calculated, it is evident that the larger the difference between observed and expected values the larger will χ^2 be, and hence the smaller the area in question. Moreover, since χ^2 is obtained by adding a series of numbers one for each class, in general, the larger the number of classes the larger the value of χ^2. Evidently the number of classes must be taken into account in finding the area under the tails. In the table of χ^2 values (Table 4.2), the quantity n in the first column is one less than the number of classes (technically n is the number of degrees of freedom, or the number of classes which can be filled up arbitrarily). In the present example there were two classes (heads and tails) and hence $n = 1$. The value 4 for χ^2 in the $n = 1$ row of the table corresponds to $P = 0{\cdot}05$ approximately. In other words, the two

TABLE 4.2
Table of χ^2
(based on Fisher (1963) by permission of Oliver and Boyd)

n	P								
	0·99	0·98	0·95	0·90	0·50	0·10	0·05	0·02	0·01
1	0·00016	0·00063	0·0039	0·016	0·46	2·7	3·8	5·4	6·6
2	0·020	0·040	0.10	0·21	1·4	4·6	6·0	7·8	9·2
3	0·12	0·19	0·35	0·58	2·4	6.3	7·8	9·8	11·3

shaded areas under the tails of the normal curve in Fig. 4.2(*b*) represent in total approximately $\frac{1}{20}$ ($\frac{1}{40}$ each) of the total area under the curve. Thus once in 20 times of throwing 100 coins one can expect to obtain a deviation from equality at least as large as 60:40. It is usual to say that a 1 in 20 probability such as this indicates a suspiciously large departure from expectation and to describe the deviation as significant. However, if one made the deduction that the coins were biassed there is a distinct possibility that one would be wrong since with unbiassed coins this deviation is equalled or exceeded once in every 20 times purely by chance. Thus a probability of 0·05 suggests further experimenting is needed, before reaching a conclusion as to whether the hypothesis of 1:1 for heads:tails is true or not. A deviation giving a P value of 0·05 is equal to approximately twice the standard deviation.

§ 4.3 The significance of Mendel's data

In Chapter 2 Mendel's results were quoted from a back-cross of heterozygous Yellow peas with green peas. The expected ratio of Yellow:green peas in the progeny is 1:1. The situation is therefore strictly comparable to the example given above of tossing coins, and the effects of chance can be assessed in the same way. Thus, in one of the experiments quoted, Mendel found 58 Yellow and 52 green peas in the progeny. It was taken for granted in Chapter 2 that this was a satisfactory fit with a 1:1 ratio, but strictly

speaking the χ^2 test ought to be applied in order to confirm this. The expected frequency in each class is 55, the deviation is 3 and so

$$\chi^2 = \frac{9}{55} \times 2 = 0{\cdot}33.$$

Reference to Table 4.2 shows that for $n = 1$, the probability of getting a deviation as large or larger than the observed one is between 0·5 and 0·9. In other words, more often than not one would expect a bigger difference from 1 : 1 purely by chance, and the data agree very well with the hypothesis.

The χ^2 test can also be applied when the expected frequencies of the classes are unequal. Thus, Mendel's F_2 data discussed in Chapter 2 are expected on his hypothesis to fit a 3 : 1 ratio. The observed frequencies for the seed colours were 6022 Yellow and 2001 green. The expected frequencies are 6017·25 and 2005·75 respectively. The deviation is 4·75 for each class and

$$\chi^2 = \frac{(4{\cdot}75)^2}{6017{\cdot}25} + \frac{(4{\cdot}75)^2}{2005{\cdot}75} = 0{\cdot}015.$$

Referring again to Table 4.2, for $n = 1$ the likelihood of getting a deviation as large or larger than that observed is approximately 0·9. Thus only once in 10 times would one expect to get so good a fit with the expected ratio.

The χ^2 method can also be used when the number of classes is more than two. Thus, Mendel's F_2 data quoted in Chapter 3 for the Round/wrinkled and Yellow/green character-differences were 315, 101, 108 and 32 in the four classes, where the expected frequencies on the hypothesis of a 9 : 3 : 3 : 1 ratio are 312·75, 104·25, 104·25 and 34·75 respectively. The deviations of the observed from the expected values are 2·25, 3·25, 3·75 and 2·75, respectively, and

$$\chi^2 = \frac{(2{\cdot}25)^2}{312{\cdot}75} + \frac{(3{\cdot}25)^2}{104{\cdot}25} + \frac{(3{\cdot}75)^2}{104{\cdot}25} + \frac{(2{\cdot}75)^2}{34{\cdot}75} = 0{\cdot}47.$$

Referring to Table 4.2, when $n = 3$, $P = 0{\cdot}90 - 0{\cdot}95$, implying a close fit with the hypothesis. Indeed 14 times out of 15 a larger deviation is expected by chance.

This finding points to a remarkable feature of Mendel's data discovered by Fisher (1936), namely, that Mendel's results taken as a whole fit the expected ratios better than they should! When the χ^2 test is applied to the results of his individual experiments, the probability values obtained usually lie between 0·5 and 0·95 as in the three examples just discussed. Individually, these are acceptable, but when one considers the data in the aggregate one's suspicions are aroused. For, with a series of experiments, the P values must on the average fall below 0·5 as often as above, assuming the data fit the hypothesis. From each experiment one reaches the conclusion that more often than not one would expect a bigger difference than that observed. Mendel's work was conducted on such a massive scale that the cumulative effect of the many experiments, each individually fitting the theory slightly better than expected on the average, is to produce an aggregate set of data which is exceedingly unlikely to be valid. Thus, Fisher found that Mendel's

total data give a P value of 0·99993, or in other words only once in some 14,300 times would one expect to get so good a fit with expectation purely by chance!

This extraordinary discovery naturally raises the question of whether Mendel in fact carried out the experiments he describes! Fisher found that many points of detail were entirely consistent with the belief that Mendel did do what he says he did. Thus, the number of plants he would have to have grown in each year was reasonable for the size of the monastery garden at Brno (Brünn); and the scoring of embryo characters in the seed, such as yellow and green, covered an extra generation compared with mature characters, like tall and dwarf, as expected since the former but not the latter character-difference can be observed without germinating the seeds.

Accepting that Mendel conducted the experiments which he describes, why did he adjust the frequencies of progeny a little nearer to the theoretical values? Here a second puzzling feature of Mendel's paper may provide a clue. Fisher has pointed out that although Mendel's first experiments were with strains of peas differing by single characters (as described in Chapter 2), yet commercial varieties of peas, with which he started, always differ by many characters. The published experiments covered five years (1859–1863 inclusive), although Mendel states that the paper is the result of eight seasons' work. It is evident that the earlier work (1856–1858 inclusive), during which the strains differing by single characters were obtained, was never published. Fisher suggests that it was on the basis of these unpublished experiments, estimated to have involved the growing of nearly 7000 pea plants, that Mendel must have framed the entire theory of heredity. At the latest this would have been when he reviewed his 1858 results. The gigantic series of experiments upon which he then embarked, involving over the succeeding five years the cultivation, in Fisher's estimation, of over 26,500 plants and forming the basis of his classic paper, is then seen as a carefully planned demonstration of the conclusions which he had already reached before the outset. The slight adjusting of frequencies would then presumably have been done in order to make the conclusions a little more obvious. In this he was singularly unsuccessful, for no-one in his lifetime saw the significance of his epoch-making discovery. Even if the adjusted figures in his paper now make the recognition of the underlying mechanism look simple, yet Mendel himself would clearly not have had this advantage when he inferred that inheritance was particulate, with segregation at gamete-formation! His brilliant discovery, probably made in the very year that Darwin's 'The Origin of Species' was published, apparently remained unknown to Darwin and was not confirmed by anyone for 40 years.

§ 4.4 Further uses of the χ^2 test

Mendel's results have been taken as an example of data which give an exceptionally high probability value with the χ^2 test. In general, P values within the range 0·1 to 0·9 cause no suspicion and indicate that the observations fit the hypothesis under test, although they may of course also fit other

hypotheses. Values of 0·05 or less are conventionally said to indicate a real discrepancy, although this is quite an arbitrary line of demarcation and one would less often be misled if one took lower figures such as 0·02 or 0·01 as the significance level. Conversely values of 0.95 or more indicate an abnormally close fit with hypothesis, while probabilities as high as 0·98 or 0·99 would certainly arouse suspicion that, either consciously or unconsciously, the data had become biassed in favour of the hypothesis.

The χ^2 test can still be used when one does not know the expected proportions of the classes, but wishes to know whether the observed frequencies are in the same proportion following different treatments. Where there are two classes a and b and two treatments 1 and 2, the four observed frequencies may be denoted by a_1, b_1, a_2 and b_2 respectively. Then χ^2 is given by the expression

$$\frac{(a_1 + b_1 + a_2 + b_2)(a_1 b_2 - a_2 b_1)^2}{(a_1 + b_1)(a_2 + b_2)(a_1 + a_2)(b_1 + b_2)} \quad \text{and} \quad n = 1.$$

The application of this formula is called the *contingency* χ^2 test, and examples of its use are given in § 7.5 and § 8.6.

If the observed frequency in any class is less than 5, the χ^2 test becomes so inaccurate as a means of estimating the area under the tails of the normal curve that it should not be used. However, Yates (1934) pointed out that when there is only one degree of freedom ($n = 1$), reasonable accuracy is restored if one adds 0·5 to the smaller observed value and subtracts 0·5 from the larger one before applying the test. With the contingency test there will be two small values and 0·5 should be added to each, and similarly there will be two large values from each of which 0·5 should be subtracted. An example showing the application of Yates' correction is given in § 4.8.

§ 4.5 Galton's and Pearson's studies on the inheritance of characters showing continuous variation

One of the first applications of statistical methods to the study of the inheritance of characters showing continuous variation was made by Galton (1889). His method was to obtain data on the attributes of relatives, notably in *Homo sapiens*, and then calculate regression and correlation coefficients. He obtained the data in several ways, but chiefly by offering prizes to anyone who would send in detailed particulars of their parents, grandparents, great-grandparents, brothers, sisters, uncles, aunts, great-uncles and great-aunts. The information asked for included stature, eye-colour, artistic aptitude, health and temperament. Data were received for about 150 families and concerned over 1000 individuals.

From the data on stature he obtained evidence of positive association between relatives. As one would expect, the parental and fraternal relationships gave the highest values for the coefficients, and cousins the lowest. He thus obtained the first sound evidence that human stature is to some extent an inherited character. Moreover, the coefficients for the two sexes did not differ significantly, indicating that their contributions to heredity were equal. From the values of the correlation coefficients he deduced that on the

average a child receives $\frac{1}{4}$ of its heritage of stature from its two parents (total $\frac{1}{2}$), $\frac{1}{16}$ from each of its four grandparents (total $\frac{1}{4}$), and so on. This geometric scale continued indefinitely backwards would account for the total heritage. He found evidence that the other attributes for which he had data were inherited similarly. Later he called this geometric series of contributions the 'law of ancestral heredity'.

Pearson (1897) extended Galton's work in a number of ways, using his data. Thus, he showed that in assessing the significance of the correlation coefficients for human stature, allowance must be made for assortive mating, for it was evident from the data that tall husbands tend to have tall wives, and short husbands short wives. For some characters the marital correlation is remarkably high: for instance, Penrose (1963) has shown that for intelligence it may be as high as 0·5. Pearson and Lee (1903) collected further data on human stature in several hundred families, by the same means as Galton had done. They also obtained data on arm-span and elbow to finger-tip distances. For stature, the parental and fraternal correlation coefficients were 0·51 and 0·54 respectively; for arm-span they were 0·45 and 0·54 respectively; and for the forearm measurements they were 0·42 and 0·46 respectively*. It will be noted that in each instance the fraternal correlation is greater than the parental. Pearson and Lee's figures for both the parental and fraternal correlation coefficients for stature are appreciably larger than those which Galton had obtained.

Galton and Pearson also made statistical studies on the inheritance of characters such as coat colour in *Canis familiaris* (Dog) and *Equus caballus* (Horse), using the information in pedigree records. They found that the correlation coefficients were similar to those in man.

§ 4.6 The multiple factor hypothesis

With the rediscovery of Mendel's work, Bateson and Saunders (1902) raised the possibility that characters showing a continuous range of variation, such as human stature, might be determined by a large number of Mendelian factors. Pearson (1904) analysed this possibility mathematically, assuming that all the postulated Mendelian factors had equal and additive effects, and that there was complete dominance of each over its recessive allele when heterozygous, and that the two alleles of a pair were equally frequent. He found that, however many pairs of alleles were postulated, the expected parental correlation was 0·33 and the fraternal 0·42. Since these values did not agree with his observed values, he concluded that the Mendelian explanation was incorrect. Yule (1907) pointed out that the assumption of complete dominance was not necessarily justified, and that if the heterozygotes were intermediate between the homozygous dominants and the recessives, higher values of the correlation coefficients would be expected, such as had been found. However, much confusion of thought on the applicability of Mendelian heredity to continuous variation persisted until Johannsen (1909)

* The weighted means are given, as calculated by Fisher (1918).

published the results of his experiments with *Phaseolus vulgaris* (Dwarf Bean) and other plants, and drew for the first time a clear distinction between the hereditary determinant, or *gene*, as he called it, and its effect.

§ 4.7 Johannsen's experiments with *Phaseolus vulgaris*

Johannsen (1903) introduced the term 'pure line' for the progeny of single individuals in organisms which are regularly self-fertilised. Examples of such organisms are *Hordeum vulgare* (Barley), *Lathyrus odoratus* (Sweet Pea) and *Phaseolus vulgaris*. He found that the weights of the seeds of the members of a pure line of *P. vulgaris* fitted a normal curve, but whether he selected heavy, medium or light beans, the progeny of each by self-fertilisation in the succeeding year again fitted a normal curve, and moreover they all had the same mean value. In other words, within a pure line selection for heavy or light seeds had no effect: the mean weight remained constant. Thus, his pure line no. 2 gave him in the year 1901 beans ranging in weight from about 40 to 70 centigrams. He classified them into four groups according to their weight (35–45, 45–55, 55–65, and 65–75 cg) and the following year he sowed each group separately. Those in the 35–45 cg class produced 86 seeds in 1902, and they showed a normal distribution about a mean weight of 57·2 cg. Those in the 45–55 cg class (1901 seed) gave 195 seeds in 1902 with a mean of 54·9 cg. Similarly the 55–65 cg class gave 120 seeds of mean weight 56·5 cg and the 65–75 cg class produced 74 seeds with mean weight 55·5 cg. The mean weights of the seeds produced by the various classes clearly do not differ significantly. He concluded that the variation in bean weight within a pure line was entirely due to environmental factors such as the position of the seed in the pod.

Johannsen studied 19 different pure lines of *Phaseolus vulgaris*. Each had been obtained from a different source, and had a characteristic mean weight of seed ranging from 64·2 cg in no. 1 down to 35·1 cg in no. 19. Within each pure line he obtained results similar to those described above for no. 2. Each gave a normal distribution of bean weight about its characteristic mean value. Within a pure line selection for heavy seeds had no effect on succeeding generations.

The total population of bean seeds under observation also gave a normal distribution, with a mean of 47·9 cg (Table 4.1 and Fig. 4.1). This population was a mixture of the 19 pure lines. Selection for heavy beans within this population would produce a response, because one would tend to pick out seeds from the heavier pure lines and hence put up the average weight the following year. Selection would continue to have an effect until all the seeds belonged to the heaviest pure line, but thereafter it would be ineffective in stepping up the mean weight further.

Johannsen's work demonstrated the distinction between variation due to hereditary factors and that due to environmental factors. He coined the term *genotype* for the hereditary constitution of an individual, and *phenotype* for the appearance of the individual as a consequence of the interaction of genotype and environment. Thus a bean seed might be heavy, say of 60 cg,

as a consequence of favourable environmental conditions during development, even though belonging to, say, pure line no. 15 with a mean weight of only 45·0 cg. Conversely, a bean of the same weight might belong to pure line no. 1 grown under rather unfavourable conditions. Both beans would be said to have the same phenotype, but the genotypic and environmental contributions to this end-result would have acted in opposite directions in the two examples. It is evident that the two kinds of variation, genetic and environmental, cannot be distinguished by observation but only by experiment. Thus, it is necessary to study the progeny of two beans before one can determine whether they belong to the same pure line or not. Differences in genotype were responsible for differences in mean seed weight of the order of 30 cg between the most extreme pure lines. This difference is of about the same magnitude as the variation in weight within pure lines due to environmental factors. It is evident that in a mixed population of a number of pure lines the variation due the the environment will wholly obscure the discontinuities in mean weight of the different lines, and give rise to a continuous range of variation.

Johannsen was able to demonstrate the respective contributions of heredity and environment to variation by choosing an organism in which, owing to the regular self-fertilisation, many individuals have the same genetic constitution. In most organisms, where cross-fertilisation is the rule, it is more difficult to distinguish the two kinds of variation. Nevertheless there is every reason to suppose that the concepts of genotype and phenotype developed by Johannsen with *Phaseolus vulgaris* are of general application. They are of particular importance in plant and animal breeding, since his work showed that selection for favourable characters in an organism will be unsuccessful unless the character-difference in question has a genetic basis and is not merely a consequence of favourable environmental conditions. Thus a first essential in crop and livestock improvement is to have available sources of hereditary variability such as strains of the organism from different regions of the world, or allied species which will give fertile hybrids with that which is being bred.

§ 4.8 Confirmation of the multiple factor hypothesis

From studies on crop plants, Nilsson-Ehle (1909) and East (1910) obtained evidence in support of the multiple factor hypothesis to explain the inheritance of quantitative characters. Nilsson-Ehle had been studying the inheritance of glume and ligule characters in *Avena sativa* (Oats) and of ear and grain pigmentation in *Triticum aestivum* (Wheat). In *Avena* he found that black glumes usually behaved as a simple Mendelian dominant to white glumes, when strains with these characters were crossed, but in one instance he obtained 630 plants with black and 40 with greyish-white glumes in F_2 from such a cross. He pointed out that this ratio approximated to 15:1 (expected frequencies 625·3 and 44·7, deviations 4·7, $\chi^2 = 0{\cdot}53$, $n = 1$, $P = 0{\cdot}5$ approximately), and so could be regarded as a 9:3:3:1 ratio in

which the first three classes looked alike. Hence he postulated that the two strains differed by two independently-inherited genes, such that each alone or both together produced a black pigment in the glumes. Similarly, with ear pigmentation in *Triticum*, a brown variety crossed with white gave brown in F_1, and usually a 3:1 ratio of brown to white in F_2, but with winter wheats he obtained 15:1 ratios in F_2.

Nilsson-Ehle also crossed strains of *Triticum* having a red pericarp (which is maternal tissue surrounding the seed) with others having a white pericarp. All the crosses gave a red F_1 and some gave 3:1 ratios of red:white in F_2. However, a cross between an old red variety called Swedish Velvet-wheat and a white variety, produced only red plants in both F_1 and F_2. This unexpected result led him to obtain an F_3 generation by self-fertilisation of the 78 F_2 plants. He found that 8 of the 78 gave a 3:1 ratio in F_3 (aggregate frequencies 307 red, 97 white); 15 gave a 15:1 ratio (total progeny 727 red, 53 white); 5 gave a 63:1 ratio (total progeny 324 red, 6 white); and the remaining 50 F_2 plants bred true, giving in total 2317 red progeny. The 63:1 ratio is what is expected if 3 independently inherited pairs of alleles are segregating simultaneously and each of the dominants *A*, *B* and *C*, has the same effect. Nilsson-Ehle suggested that there were three such dominant genes responsible for the red pericarp colour in Velvet-wheat and that the white variety with which it was crossed carried the recessive alleles *a*, *b* and *c*. One would then expect a 63:1 ratio in F_2, with which the 78:0 ratio observed is in satisfactory agreement. (For the χ^2 test it is necessary to apply Yates' correction, since one of the observed frequencies is less than 5. The 'corrected' observed frequencies are 77·5 and 0·5, the expected frequencies on the 63:1 hypothesis are 76·8 and 1·2 respectively. Hence the deviations are 0·7 each, $\chi^2 = 0{\cdot}41$, $n = 1$ and $P = 0{\cdot}5$ approximately.) Moreover, Nilsson-Ehle showed that on his three gene hypothesis the expectations in F_3 agree satisfactorily with what he had found. On the average, out of every 63 red F_2 plants, 6 are expected to give 3 reds:1 white in F_3, 12 to give a 15:1 ratio, 8 to give a 63:1 ratio and the remainder to breed true.

Nilsson-Ehle suggested that the hereditary basis of continuous variation might be similar to what he had found in *Avena* and *Triticum* for discontinuous variation. In other words, if there were a number of genes affecting the same character, such that the discontinuities of phenotype were sufficiently small to be obscured by environmental variation, then continuous variation would be manifest.

East (1910) had studied the inheritance of various grain characters and of the number of rows of grains on the ear in *Zea mays*. Some of the grain characters gave 15:1 ratios in F_2 suggesting control by two independently-inherited pairs of alleles, but the number of rows of grains on the ear showed continuous variation. East found that there was always an even number of rows, and that the number of pairs of rows varied from 4 to 15. He made a number of crosses between strains differing in mean number of pairs of rows and studied the progeny. For example, he crossed a strain of *Zea* in which 28 ears had a mean of 4·14 pairs of rows and standard deviation 0·45 with one in which 107 ears had a mean of 6·59 pairs and standard deviation

0·79*. He obtained 21 F_1 ears with a mean of 5·62 pairs of rows and standard deviation 0·51. In other words, the F_1 was intermediate between the two parents and relatively uniform. He selected an F_1 ear with 6 pairs of rows and obtained 77 F_2 ears by self-fertilisation. These had a mean of 5·44 pairs of rows and standard deviation 0·82. Thus, although the F_2 mean was similar to that of the F_1, the F_2 plants were much more variable. East pointed out that this tendency to recover in F_2 some individuals that approached the parental forms in appearance is what would be expected if segregation of several pairs of alleles controlling row number was taking place, and hence he favoured the multiple factor hypothesis of inheritance of characters showing a continuous range of variation.

TABLE 4.3 Emerson and East's data for the frequencies of maize ears of various lengths from a cross between a short-eared and a long-eared strain.

Generation	No. of Ears Measd.	Ear Length to Nearest Centimetre																	Mean Lgth. cm.	Standard Deviation cm.
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
P	57	4	21	24	8														6·6	0·8
P	101									3	11	12	15	26	15	10	7	2	16·8	1·9
F_1	69					1	12	12	14	17	9	4							12·1	1·5
F_2	401			1	10	19	26	47	73	68	68	39	25	15	9	1			12·9	2·3

An essential feature of this multiple factor hypothesis is that the effects of the different genes should be cumulative. If they merely duplicated one another, discontinuous variation would result, with F_2 ratios of 15:1, 63:1, and so on, as in the examples of these ratios found by Nilsson-Ehle and East. However, if the phenotype depends on the dosage of a series of different genes, then even with quite a small number of them, a considerable number of alternative phenotypes becomes possible, leading to greatly increased variability in the F_2 generation from a cross between two pure-breeding lines.

This increased variability in F_2, which Kölreuter (1763) was the first to discover, and which East (1910) had found, has since been amply confirmed for numerous characters showing continuous variation and in a great diversity of organisms. One of the clearest examples was from the work of Emerson and East (1913). They had studied the inheritance of numerous quantitative characters in *Zea mays*, such as lengths and diameter of ears, weight of grains, height of plant, number of nodes, internode length and duration of growth. From a cross between two strains differing markedly in ear length, the results shown in Table 4.3 and Fig. 4.3 were obtained. The

* From such data as these, it is possible to calculate the *standard error* of the mean, which is a measure of the precision of the estimate of the mean. The standard error of the mean is obtained by taking the standard deviation of the sample and dividing it by the square root of the number of observations. Thus the first parental strain has a mean of 4·14 ± 0·09 pairs of rows, since its standard error is $\frac{0{\cdot}45}{\sqrt{28}} = 0{\cdot}09$, and similarly the second parental strain had a mean of 6·59 ± 0·08 pairs of rows, since its standard error is $\frac{0{\cdot}79}{\sqrt{107}} = 0{\cdot}08$. The first mean does not differ significantly from 4, since once in twenty times one would expect a deviation of plus or minus twice the standard error or more. By a similar argument one would deduce that the second mean differs significantly from both 6 and 7.

short-eared parent was relatively uniform with a mean ear-length of 6·6 cm and standard deviation 0·8 cm. The long-eared parent was more variable (mean length 16·8 cm, standard deviation 1·9 cm). The F_1 plants were intermediate and relatively uniform (mean length 12.1 cm, standard deviation 1·5 cm) while the F_2 plants were much more variable (mean length 12·9 cm, standard deviation 2·3 cm).

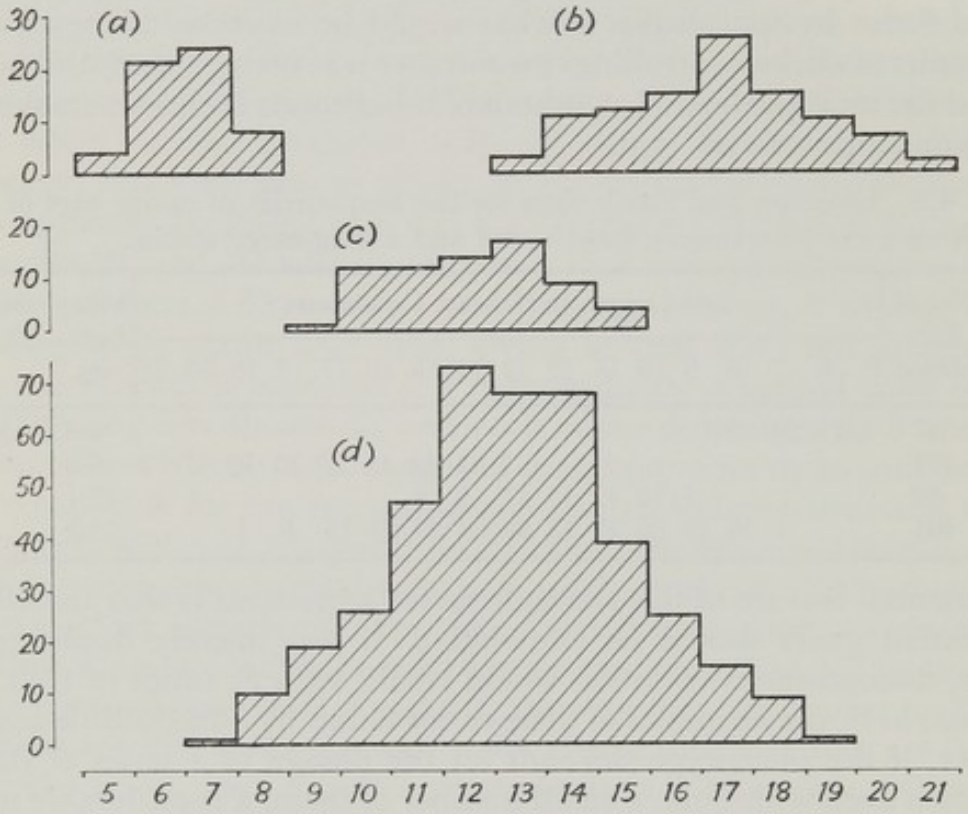


FIGURE 4.3 Frequency histograms for ear length in *Zea mays* from the data of Emerson and East. The number of ears is plotted as ordinate and the ear length in centimetres as abscissa. (*a*) and (*b*) represent the two parental strains, (*c*) the F_1 generation, and (*d*) the F_2 generation.

Fisher (1918) took up the problem of testing the agreement between the observed values of the correlation coefficients between relatives and those expected on the multiple factor hypothesis, without making the arbitrary simplifications which had misled Pearson. Fisher made no assumptions about equality of effect of the various factors, nor about dominance or equality of frequency between alleles. He began by pointing out that when there are two or more independent causes of variability in a population, their contributions to the variance are additive. This necessarily implies that their contributions to the standard deviation are not additive. Hence, in considering the causes of variation in a population it is desirable to use the variance, rather than the standard deviation, as a measure of the dispersion about the mean value. He interpreted the fraternal correlation coefficient for human stature of 0·54, which Pearson and Lee had obtained, by saying that 54% of the variance of brothers is accounted for by ancestry. On the hypothesis of a

large number of Mendelian factors determining stature, the greater part of the remaining 46% of the variance would be due to the segregation of factors for which the parents were heterozygous, and a small part to environmental differences during the brothers' childhood.

Fisher confirmed Yule's finding that the effect of dominance between the alleles is to reduce the parental correlation coefficient by a certain amount. But Fisher took the analysis much further and showed that the effect of dominance is to reduce the fraternal correlation to only half the extent to which the parental correlation is reduced. This enables the effects of dominance to be distinguished from the effects of environmental factors, and moreover explains why the fraternal correlation coefficient exceeds the parental. The observation of this excess by Pearson and Lee is thus in itself evidence in favour of the theory of cumulative Mendelian factors, and moreover with a considerable amount of dominance between the alleles. Fisher estimated that not more than 5% of the fraternal variance was due to environmental factors. His estimates for the correlation coefficients between relatives on the basis of the multiple factor hypothesis were in excellent agreement with the observations of Pearson and Lee, and others. Moreover, on making certain assumptions about assortive mating Fisher was able to deduce Galton's 'law of ancestral heredity'. Thus the controversial question of how far the inheritance of continuous variation could be attributed to Mendelian factors was finally resolved.

Much further support for the multiple factor theory has been obtained subsequently. Fisher's idea of partitioning the variance between the various causes of variability has been greatly extended, and has led to a much fuller understanding of the genetics of quantitative characters. Thus, Mather (1949*a*) has demonstrated that the genes responsible for the inheritance of continuously variable characters are not all inherited independently, but may show linkage, comparable to that known for certain genes controlling discontinuous characters. The method of establishing linkage is based on the analysis of variance. Consider two inbred lines differing in a quantitative character such as abdominal bristle number (*Drosophila*), or height or length of parts (plants), and assume that this character is determined by numerous genes. The character is counted or measured in a number of individuals of each line, and the means and variances calculated. Assuming that the two strains have each been inbred for a number of generations, the individuals of each will be almost uniform genetically, like Johannsen's pure lines of *Phaseolus*. In consequence, the observed variance can be attributed almost entirely to environmental factors. The two lines are then crossed and a number of individuals of the F_1 measured. The mean value for the character is expected to be intermediate in the F_1 between the two parents, as East (1910) and Emerson and East (1913) had found. Moreover, the variance of the F_1 will be due wholly to environmental factors, since all the individuals will have the same genotype, being heterozygous for the alleles by which the parents differ. An F_2 generation is raised by self-pollination (plants) or inter-crossing of F_1 individuals, and again the mean and variance are calculated from measurements of a number of individuals. The variance

will now have both genetic and environmental components, since segregation of alleles will have taken place. As a first approximation it may be assumed that the environmental variance is constant in the parental, F_1 and F_2 generations, if they have been grown under similar conditions, and hence the genetic contribution to the F_2 variance can be estimated. However, recent work has indicated that the F_1 generation commonly has a smaller variance than the initial inbred lines, indicating that the development of the latter is more readily modified by the environment. Some of the F_1 individuals are

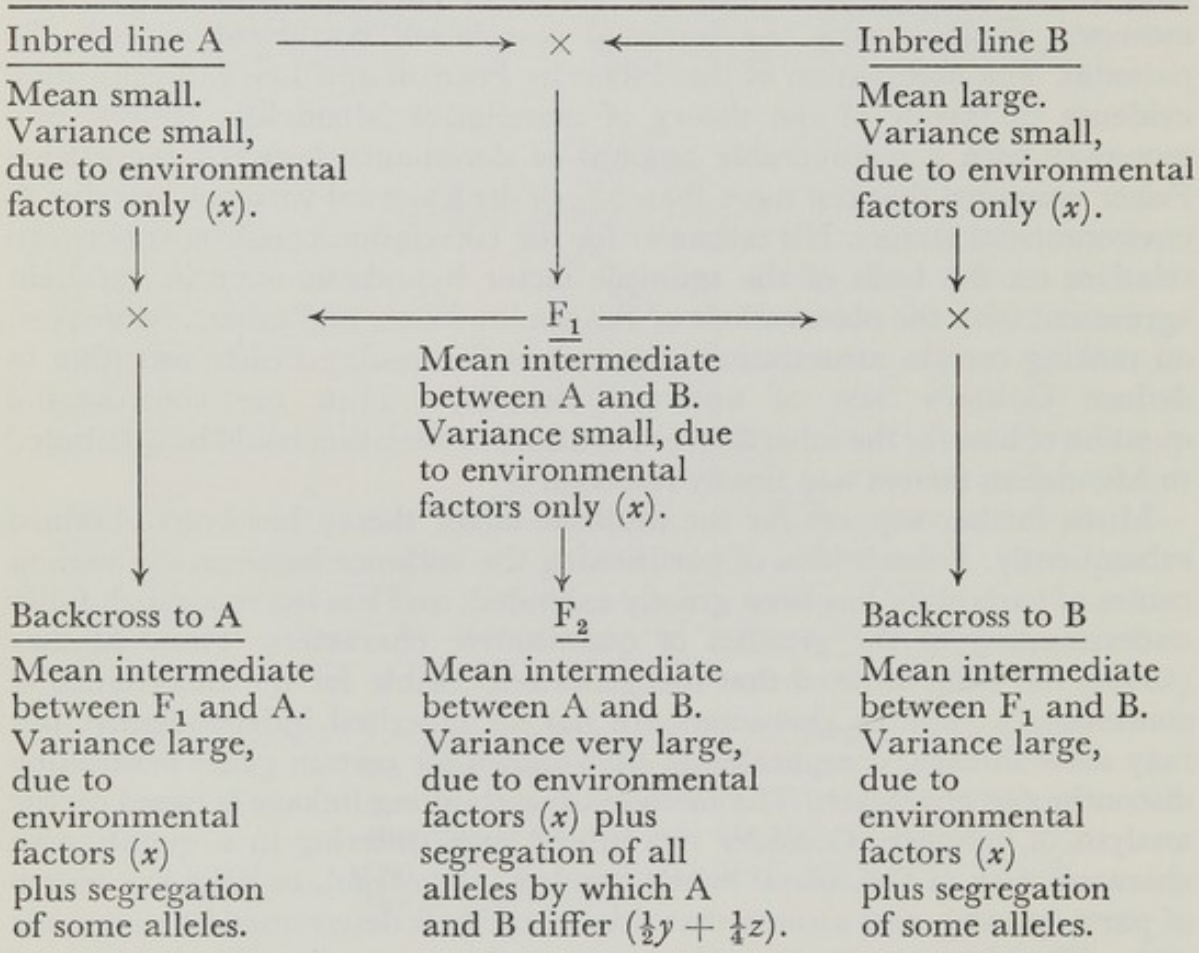


FIGURE 4.4 Diagram showing the means and variances expected when two inbred lines are crossed and the progeny inbred and also backcrossed. It is assumed that the environmental conditions are constant, that each population responds similarly to environmental effects (not necessarily true—see text), and that the character under study shows continuous variation determined by a number of independent Mendelian factors.

x = environmental contribution to variance.

y = measure of difference in phenotype between corresponding homozygotes, such as AA and aa.

z = measure of dominance, that is, difference in phenotype between AA and Aa and between Aa and aa, and similarly with the other factors.

back-crossed to each parent, and the means and variances of the progeny determined. As with the F_2, the back-cross progeny will have genetic and environmental contributions to the variance, since the alleles by which the inbred lines differed will also segregate in the back-crosses.

The hereditary component of variance can be considered to consist of two parts. One is concerned with the magnitude of the effect of the various genes, that is, the difference of phenotype between *AA* and *aa*, and similarly for all the other pairs of alleles affecting the character. The other part is concerned with dominance, that is, how far the phenotype of *Aa* is intermediate between *AA* and *aa*, and similarly for the other pairs of alleles. If one compares the average variance of the progenies from back-crossing to each parent with the variance of the F_2, the environmental effects may be considered as a first approximation to contribute equally to each, and similarly with the dominance effects (*Aa versus AA* and *aa*). On the other hand, the homozygote differences (*AA versus aa*) will contribute much less to the average variance of the backcrosses than to the F_2 variance, because in the back-crosses one cannot have both homozygotes for a pair of alleles (for example, *AA* and *aa*) present at once. This explains why the variance of the back-crosses is less than that of the F_2 (see Fig. 4.4). From a comparison of the average variance of the backcrosses with the F_2 variance, the respective contributions of homozygote differences and of dominance can be estimated. In most experiments the former considerably exceeds the latter.

From the analysis of variance in F_3, further estimates can be obtained of the size of the various components of the variance. From studies of the variance for plant height in inbred lines of *Antirrhinum majus* and *A. glutinosum* and of the F_1, F_2, F_3 and back-cross progenies, Mather (1949*b*) found internal discrepancies in the various estimates for the components of the variance. However, by making allowance for the possibility of linkage between some of the genes, an excellent agreement with observation was obtained. He deduced that linkage was present. Confirmation of linkage between genes responsible for continuously variable characters has been obtained, for example in *Drosophila*, by studying quantitative characters in conjunction with one or more discontinuous characters.

§ 4.9 Conclusion

The hypothesis of multiple Mendelian factors, each with additive effects, has been found satisfactorily to explain data on the inheritance of many quantitative characters. The phenomena of dominance and of linkage, which are manifest in Mendelian inheritance of discontinuous characters, have also been demonstrated with characters showing a continuous range of variation. There is no reason, therefore, to suppose that the inheritance of quantitative characters differs in any fundamental way from that of qualitative characters which show Mendelian inheritance. There is evidence, however, that at least some of the genes affecting quantitative characters may occur in special regions of the hereditary material (see Chapter 18).

5. *The theory that Mendelian inheritance is determined by the cell-nucleus and non-Mendelian by the cytoplasm*

§ 5.1 Weismann's theory of the continuity of the germ-plasm

The term *nucleus* was applied by Brown (1833) to the more or less spherical structure which usually occupies a central position in cells. He observed the nucleus in the cells of both the vegetative and reproductive parts of many flowering plants. He made particular mention of its occurrence in the leaf-cells of orchids and in the stigma and pollen of *Tradescantia*. The fate of the nucleus in cell-multiplication was a source of debate and speculation until Strasburger (1879) reached the conclusion that nuclei arise only from pre-existing nuclei. He had made extensive studies of the cells of the reproductive organs of numerous seed-plants. Meanwhile, Hertwig (1875) with *Paracentrotus lividus* (a species of Sea Urchin) and van Beneden (1875) with *Oryctolagus cuniculus* (Rabbit) had independently established that at fertilization there occurs not merely a fusion of cells but also of nuclei, and moreover these are derived from the egg and the sperm, respectively. Strasburger's discovery that the cell-nucleus has itself the property of heredity, in that it never arises afresh but is always handed down, led naturally to the conclusion that the nucleus is the vehicle of heredity.

It was against this cytological background that Weismann (1883, 1885) put forward his theory of the continuity of the germ-plasm. The essence of this theory was 'that heredity is brought about by the transference from one generation to another, of a substance with a definite chemical, and above all, molecular constitution' (1885, p. 168). He called this substance 'germ-plasm', and he supposed that in the development of the body or 'soma' of an individual from the fertilised egg-cell, this substance, or part of it, was 'reserved unchanged for the formation of the germ-cells of the following generation' (1885, p. 168). This notion had a considerable influence on contemporary thought. Weismann (1883) was the first person to challenge the existence of the inheritance of mutilations, or 'acquired characters', as they were called by Lamarck (1809), an idea which, as indicated in Chapter 1, had been widely accepted ever since the time of Hippocrates. For it was fundamental to Weismann's theory that this chemical (the germ-plasm) could not be

modified by the environment during the life of an individual, but was handed down unaltered. Weismann accepted the opinions of Hertwig and Strasburger that 'the nature of heredity is based upon the transmission of nuclear substance with a specific molecular constitution' (Weismann 1885, p. 180) and went on to equate this substance with his postulated germ-plasm.

§ 5.2 Boveri's experiments with echinoderms

Strong support for the view that the nucleus is the primary carrier of hereditary material in the cell was obtained by Boveri (1889) from studies on echinoderms. Working at the Zoological Station at Naples, he added sea urchin sperm to eggs which had been fragmented by shaking, and he found that even pieces of egg lacking a nucleus could sometimes be fertilised. The resulting larvae were quite normal in appearance, except that they were only about one-quarter of the size of those developed from unfragmented eggs or from fragmented eggs with a nucleus. He deduced from this experiment that

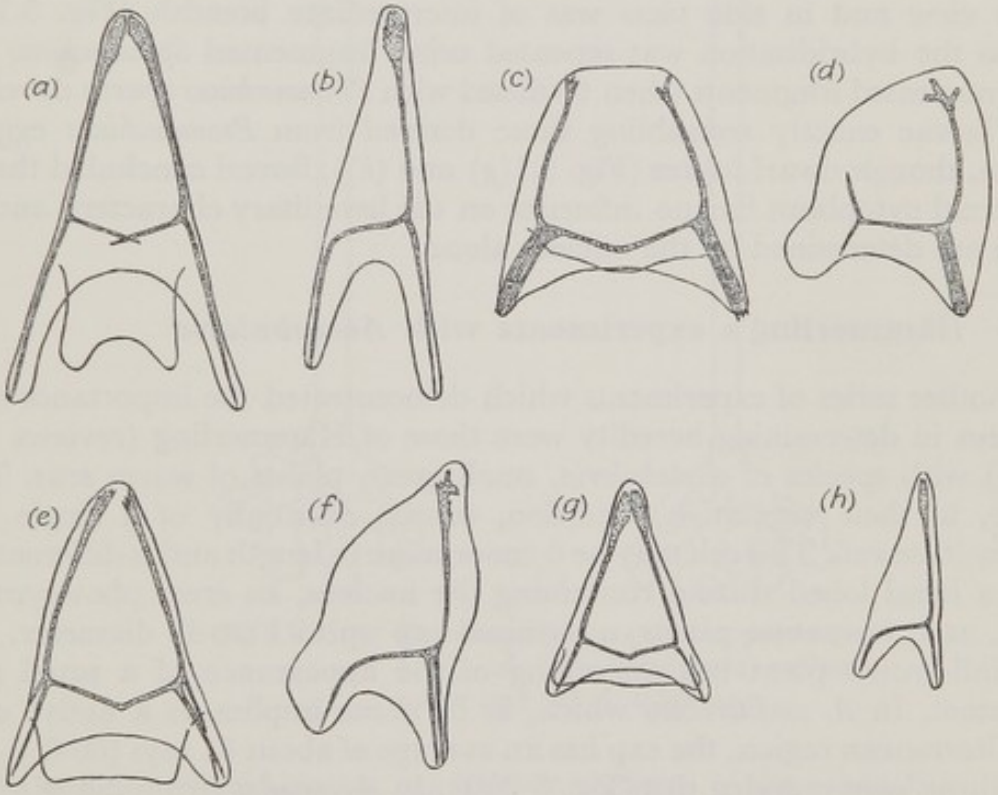


FIGURE 5.1 Posterior and side views (×100) of the calcereous skeletons of the larvae of sea urchins (from Boveri, 1889).
(*a*) and (*b*) *Psammechinus microtuberculatus*
(*a*) Posterior view (*b*) Side view
(*c*) and (*d*) *Sphaerechinus granularis*
(*c*) Posterior view (*d*) Side view
(*e*) and (*f*) *S. granularis* × *P. microtuberculatus*
(*e*) Posterior view (*f*) Side view
(*g*) and (*h*) *S. granularis* (enucleated) × *P. microtuberculatus*
(*g*) Posterior view (*h*) Side view

the sperm nucleus possessed all the properties necessary to function like a fertilised egg-nucleus. This was an important conclusion as it implied that the egg-nucleus and the sperm-nucleus were essentially alike in their hereditary contributions.

Boveri made further experiments with even more striking results. He hybridised two species of sea urchin, *Psammechinus microtuberculatus* and *Sphaerechinus granularis*. The calcareous skeleton of the *Psammechinus* larva when seen in posterior view is shaped like a capital A of rather narrow outline (Fig. 5.1(*a*)) and the body is similarly shaped. In side view, the appearance is even narrower (Fig. 5.1(*b*)). On the other hand, the *Sphaerechinus* larva is much broader, the skeleton in posterior view almost resembling a capital H (Fig. 5.1(*c*)), but with the uprights slightly tilted towards the A shape. The body is correspondingly broad, and is almost as wide in side view (Fig. 5.1(*d*)). Boveri found that when normal *Sphaerechinus* eggs were fertilised by *Psammechinus* sperm, the resulting hybrid larvae were almost exactly intermediate in appearance between those of the two parents. The skeleton in posterior view was like a capital A of broad outline (Fig. 5.1(*e*)), and the body both in front view and in side view was of intermediate breadth (Fig. 5.1(*f*)). When the hybridisation was repeated using fragmented *Sphaerechinus* eggs, the enucleated fragments when fertilised with *Psammechinus* sperm developed into larvae exactly resembling those derived from *Psammechinus* egg and sperm, though dwarf in size (Fig. 5.1(*g*) and (*h*)). Boveri concluded that the maternal cytoplasm has no influence on the hereditary characters, and that these are determined by the nucleus alone.

§ 5.3 Hämmerling's experiments with *Acetabularia*

Another series of experiments which demonstrated the importance of the nucleus in determining heredity were those of Hämmerling (reviews 1953, 1963) with species of *Acetabularia*, small green plants of warm seas. These algae, in their vegetative condition, consist essentially of a single giant uninucleate cell. This cell may be 6 cm or more in length and is differentiated into a basal lobed rhizoid containing the nucleus, an erect photosynthetic stalk, and, in mature plants, a terminal cap up to 1 cm in diameter. Thus the full-grown plant has something of the appearance of a small green toadstool. In *A. mediterranea* which, as its name implies, is a native of the Mediterranean region, the cap has an average of about 81 rays ($80{\cdot}8 \pm 3{\cdot}2$), and these have rounded tips (Fig. 5·2(*a*)). In *A. crenulata* from the W. Indies there are only, on average, 31 rays ($30{\cdot}7 \pm 2{\cdot}8$) per cap, and the tips of the rays are pointed (Fig. 5.2(*b*)).

Hämmerling took immature plants of each species and grafted them together by cutting off the tops of the stalks (the caps had not yet developed) and bringing the cut surfaces into contact. A new stalk soon developed at the point of grafting, and this gave rise to a cap which was intermediate in appearance between the two parents. A number of such caps had an average of about 42 rays ($42{\cdot}3 \pm 4{\cdot}8$) and hence approached *A. crenulata*, but with tips usually rounded like *A. mediterranea*. The intermediate character of this

graft-hybrid is to be expected, since it contains two nuclei, one from each species. Control experiments in which two plants of *A. mediterranea* were grafted together gave caps resembling this species. Corresponding results were obtained with the other species.

Further grafts were achieved using the stalk of an immature plant of one

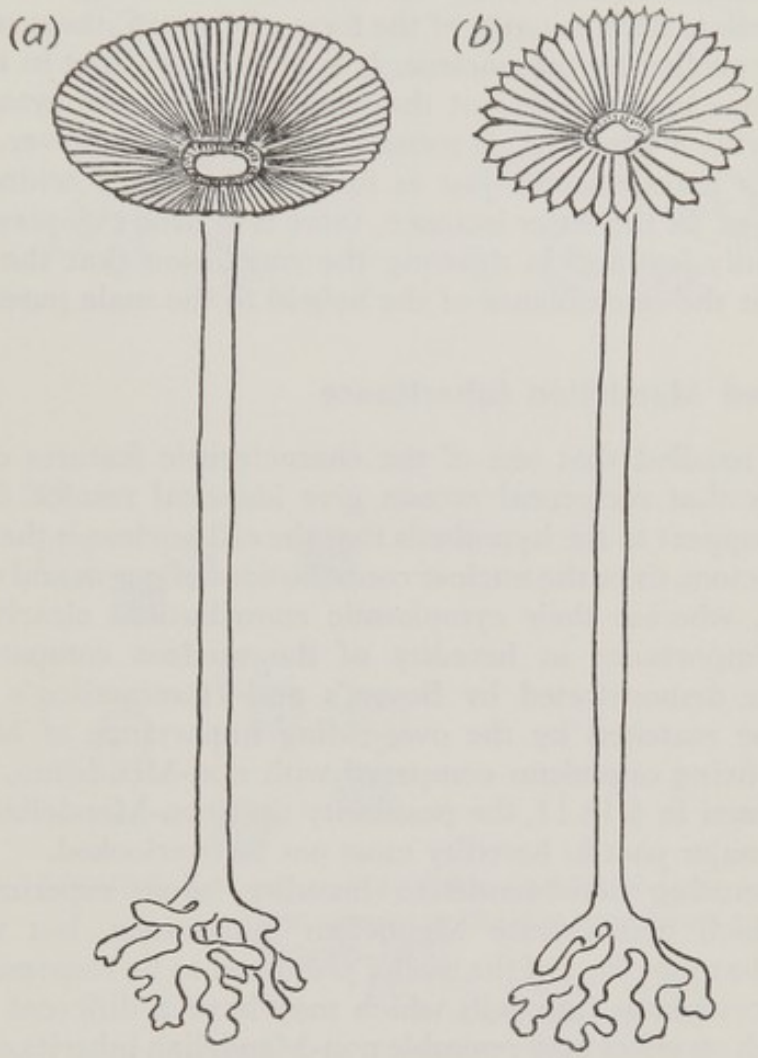


FIGURE 5.2 Drawings to show the appearance (×2) of (*a*) *Acetabularia mediterranea*, and (*b*) *A. crenulata*.

species and the rhizoid system (and hence nucleus) of the other. Such grafts contained only one nucleus. The type of cap which developed was found to depend on the length of stalk grafted. If it was about 1 to 2 cm. long, the cap was often of intermediate appearance between that of the two species, whereas if the piece of the stalk that was grafted on was about 0·5 cm long or less, the cap usually resembled that of the rhizoid (and hence nuclear) parent. It was noteworthy that, however long the stalk, the cap never wholly resembled the species from which the stalk was obtained. Hämmerling deduced that the nucleus in the rhizoid had an over-riding control over the form of the cap, but that the stalk also had some influence.

This result is not so clear cut as that obtained by Boveri with echinoderms. However, Hämmerling found that if in these graft-hybrids of the rhizoid of one species of *Acetabularia* with the stalk of the other, he cut off the first cap to form, in due course a second would develop and this invariably resembled the cap of the species which provided the rhizoid (and hence the nucleus). Hämmerling concluded that although substances in the cytoplasm are the immediate determinants of the form of the cap, these substances are ultimately controlled by the nucleus. It is to be noted that in these grafting experiments one cannot rule out the possibility that the cytoplasm in the rhizoid is playing a vital part. It seems more probable however, that it is the nucleus which is responsible, just as in Boveri's hybrid echinoderms from enucleated eggs. In the latter instance, there is so little cytoplasm in a sperm that one is fully justified in drawing the conclusion that the nucleus was responsible for the resemblance of the hybrid to the male parent.

§ 5.4 Delayed Mendelian inheritance

It will be recalled that one of the characteristic features of Mendelian inheritance is that reciprocal crosses give identical results. Such identity gives strong support to the hypothesis that the cell nucleus is the bearer of the Mendelian factors, since the nuclear contributions of sperm and egg appear to be the same, whereas their cytoplasmic contributions clearly differ. The over-riding importance in heredity of the nucleus compared with the cytoplasm, as demonstrated by Boveri's and Hämmerling's experiments, appears to be matched by the over-riding importance of Mendelian inheritance in living organisms compared with non-Mendelian. However, as will be discussed in § 18.11, the possibility that non-Mendelian inheritance does play a major part in heredity must not be overlooked.

Before discussing non-Mendelian heredity, some experiments will be described which demonstrate Mendelian inheritance, but with delayed action such that the effect of the nuclei presumed to be responsible is carried over in the cytoplasm into cells which may have a different nuclear constitution. Such an effect may resemble non-Mendelian inheritance, and needs to be carefully distinguished from it.

One example of such delayed Mendelian heredity has already been described in Chapter 3, namely, the inheritance of pollen shape in *Lathyrus odoratus* studied by Bateson, Saunders and Punnett (1905). Although segregation of factors must occur before the formation of the pollen grains, as first postulated by Mendel, yet when a pure-breeding strain of *Lathyrus odoratus* with long pollen-grains (Fig. 5.3(*a*)) is crossed with a pure-breeding strain with round grains (Fig. 5.3(*b*)), the F_1 plants as already mentioned have pollen grains which are all long in shape, and in F_2 three-quarters of the plants have long pollen and the remaining one-quarter have round pollen. It is evident from these results that long pollen-shape is determined by a dominant factor (*L*) and round pollen-shape by the recessive allele (*l*). It follows that the F_1 plants have the genetic constitution *Ll* and half their pollen grains must contain the *L* factor and the other half the *l* factor.

Nevertheless, all the grains from these plants have the *L* shape. It is evident that pollen shape must be controlled by the genetic constitution of the anther and not by that of the pollen grain itself. The simplest hypothesis is that some substance determining pollen shape is produced by the nuclei of the pollen

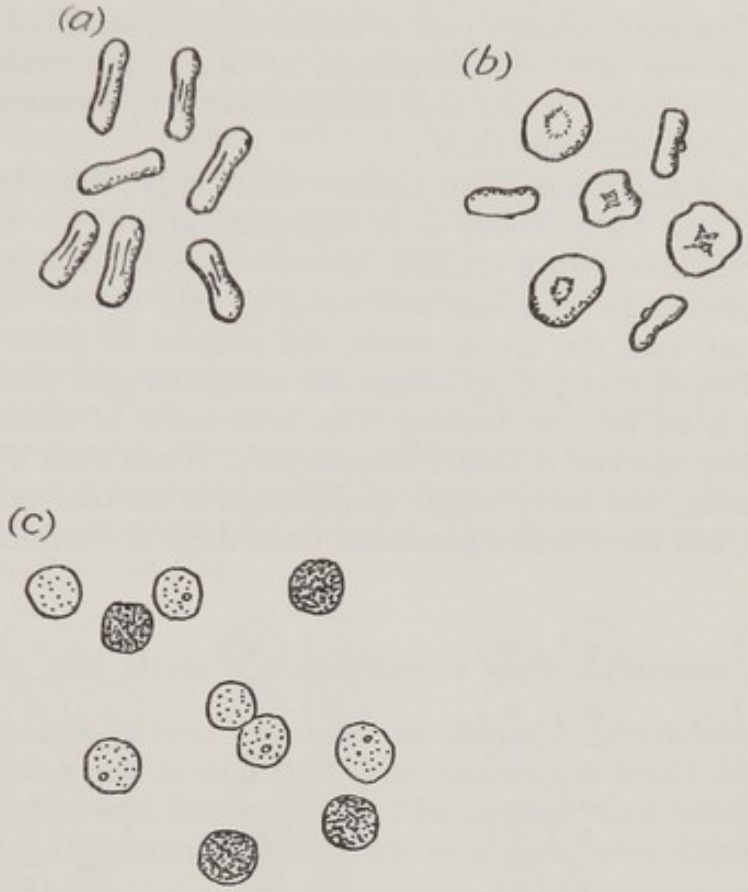


FIGURE 5.3 (*a*) and (*b*) Pollen (×175) of *Lathyrus odoratus* in the dry state (from Bateson, 1909).
(*a*) The normal Long (cylindrical) pollen-grains (usually with three germ-pores)
(*b*) The mutant round (disc-shaped) pollen-grains (usually two-pored)
(*c*) Pollen (×125) of *Oryza sativa* segregating for starchy (shown dark) and waxy (shown light) contents (from Parnell, 1921).

mother cells (or other anther cells) and carried over to the developing pollen-grains in the cytoplasm. This delayed manifestation of a nuclear effect is comparable to that found by Hämmerling in *Acetabularia* species grafts, where the second cap to form resembles that of the parent contributing the nucleus, whereas the first cap is more often intermediate. It is known that other characters of pollen in flowering plants may not show this

delayed inheritance. Thus, Parnell (1921) discovered that in *Oryza sativa* (Rice) if a pure-breeding strain with normal starchy pollen-grains (staining blue with iodine) is crossed with a pure-breeding strain with waxy pollen-grains containing erythrodextrin (staining reddish-brown with iodine), the F_1 plants have half their pollen-grains starchy and the other half waxy (Fig. 5.3(*c*)). Evidently a single pair of alleles determines this difference, and the factors function after the formation of the pollen grains. A similar situation was discovered in *Zea mays* independently by Demerec (1924) and by Brink and MacGillivray (1924).

A second example of a delayed nuclear effect is provided by the work of Gossop, Yuill, and Yuill (1940) with *Aspergillus niger*. This asexual fungus normally has chains of black conidia (asexual spores), but a strain is known with cinnamon-coloured conidia and another with brown conidia. If the cinnamon strain and the brown strain are allowed to grow together, the hyphae may fuse so that the mycelium has within its cells nuclei from both strains (there is no nuclear fusion). The occurrence of nuclei of different genotype within one cell is called *heterokaryosis*. When such a heterokaryon produces conidia, the erect aerial conidiophores are composed of multinucleate cells, but the conidia produced from these are uninucleate. Hence

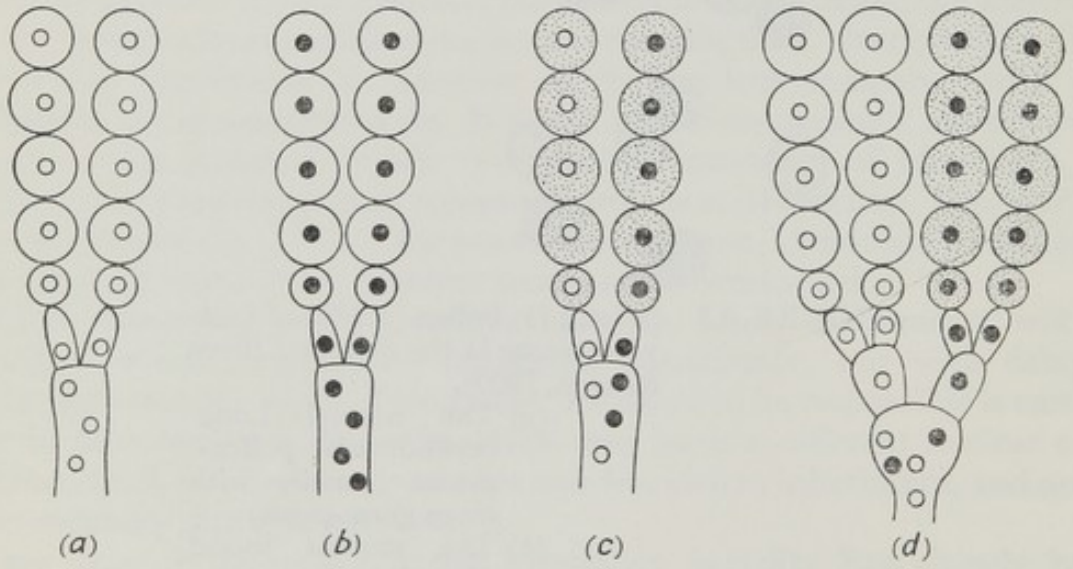


FIGURE 5.4 Conidia (×2000) of *Penicillium* and *Aspergillus* (from Pontecorvo, 1947).

(*a–c*) *Penicillium notatum*. Small open circles indicate nuclei carrying the gene for white conidia, solid circles those carrying the gene for yellow conidia, and dots in the cytoplasm indicate synthesis of the normal green conidial pigment. (*a*) White mutant. (*b*) Yellow mutant. (*c*) White-yellow heterokaryon with green conidia.

(*d*) *Aspergillus nidulans*. Small open circles indicate nuclei carrying the gene for white conidia, solid circles those carrying the normal allele for green conidia, and dots in the cytoplasm indicate synthesis of the green pigment. The white-green heterokaryon has some chains of spores white and others green.

in the formation of conidia, the heterokaryon breaks down and each conidium receives either a 'cinnamon' or a 'brown' nucleus. Nevertheless, all the conidia are black. Evidently the black pigment, or a precursor of it, is manufactured in the heterokaryotic conidiophore, where both types of nuclei can co-operate in its production, and is then transmitted in the cytoplasm to the conidia. The colonies derived from these conidia, if isolated, will be either cinnamon or brown in conidial colour according to the nuclei they have received. This is comparable to the long and round pollen shapes in *Lathyrus odoratus*. An even more striking example was described by Pontecorvo and Gemmell (1944), where white-spored and yellow-spored mutants of the normally green-spored *Penicillium notatum* were found to produce a heterokaryon which had green conidia, although these are uninucleate. When isolated, these conidia produced strains with either white or yellow conidia (Fig. 5.4(*a*)–(*c*)).

Other characters in fungi may not show this delay in the manifestation of the effects of nuclei. Thus, Gossop, Yuill, and Yuill found that in *Aspergillus nidulans* a heterokaryon between a white-spored mutant and the normal green-spored fungus produced mixed heads in which single chains of conidia were either green or white (Fig. 5.4(*d*)). This is analogous to starchy and waxy pollen in *Oryza* or *Zea*.

§ 5.5 Boycott and Diver's experiments with *Limnaea*

One of the best-known examples of delayed Mendelian inheritance concerns the direction of coiling in the fresh-water snail *Limnaea peregra* studied by Boycott and Diver (1923). The normal snail has the body and shell coiled in a right-handed spiral but in a pond near Leeds, Yorkshire,

FIGURE 5.5 *Limnaea peregra* (×2·5). (*a*) Normal dextral snail shell. (*b*) Sinistral snail shell.

along with the normal form another was found which was an exact mirror image of it (Fig. 5.5). Not only were the body and shell coiled in a left-handed spiral, but the positions of the heart and kidney were complementary to those in the normal animal, and the alimentary and reproductive openings

were on the left instead of the right side of the neck. Earlier work by other authors had indicated that the difference between dextral and sinistral snails becomes apparent at the earliest stage of development, since the plane of the first cell-division in the fertilised egg is different in the two forms.

Limnaea peregra is hermaphrodite, and although cross-fertilisation is usual, self-fertilisation can also occur. Individuals which had not yet reached sexual maturity were isolated and allowed to fertilise themselves. Broods of several hundred snails were obtained in this way from each of 144 individuals, all of related ancestry. It was found that, apart from rare exceptions which were possibly due to environmental factors, all the snails in a brood were alike as regards their direction of coiling, but either type of parent (dextral or sinistral) could produce either type of brood!

Boycott and Diver explained these remarkable results by postulating that 'the appearance of the individual is the result of, and is determined by, the product of the parental nuclear composition . . .' (1923, p. 211). Sturtevant (1923), on the basis of their results, was more specific and suggested that, in view of the very early development of the difference between dextral and sinistral forms, it was likely to be the nuclear composition of the mother which determined the direction of coiling. Two further observations made by Boycott and Diver, namely, that sinistral snails obtained by self-fertilisation bred true, whereas dextral snails similarly obtained often did not, led Sturtevant to postulate that the dextral condition was dominant to sinistral.

Later work by Boycott, Diver, Garstang and Turner (1930) confirmed both of Sturtevant's postulates. In crosses between pure-breeding dextral and pure-breeding sinistral forms, the appearance of the F_1 progeny was the same as their mother, the F_2 snails were all dextral, and in F_3 1192 dextral and 401 sinistral broods were obtained. These results are intelligible as simple Mendelian inheritance, provided the direction of a snail's coiling is always regarded as a property of its mother. Evidently the mother's nuclear constitution leads to the production of some substance in the cytoplasm of the cells of the ovary or primary oocyte, and this substance reaches the next generation by way of the cytoplasm of the egg, where, after fertilisation, it determines the plane of the first cell-division, and hence the dextrality or sinistrality of the offspring.

§ 5.6 Kühn's experiments with *Ephestia*

A rather similar example of delayed Mendelian inheritance concerns eye-colour in the moth *Ephestia kuhniella*. The eyes are normally black but a variant with red eyes is inherited as a Mendelian recessive. Kühn (1937) discovered that if red-eyed females (*aa*) are crossed with heterozygous black-eyed males (*Aa*), the black-eyed (*Aa*) and red-eyed (*aa*) progeny can be distinguished even in the young larvae. However, when the reciprocal cross is made, all the young larvae have black eyes, but in the individuals of constitution *aa* the colour gradually changes to red as they develop. Evidently a precursor of the pigment is carried via the cytoplasm of the egg to the progeny, but its effect does not persist to adult life, except in those progeny

(*Aa*) which carry the black-eye factor and hence can renew production of the precursor and pigment. Kühn confirmed this hypothesis by grafting testes from a black-eyed (*Aa*) larva into a red-eyed (*aa*) female one. It then developed a darker eye-colour which also appeared in the young *aa* progeny larvae. The substance which was shown by these experiments to be carried over in the cytoplasm of the egg to the next generation has been identified as kynurenine, which is also a precursor of *Drosophila* eye-pigment.

§ 5.7 Spurway's experiments with grandchildless *Drosophila*

A more extreme example of delayed Mendelian inheritance is provided by the mutant called *grandchildless* described by Spurway (1948) in *Drosophila subobscura*. During breeding work with this species of fly, all the males in a particular family were found to have abnormally small internal reproductive organs. These organs have an orange pigment and can be seen in living flies through the body wall. On dissection no testes or only the minutest rudiments were found. The same condition appeared in the progeny of sister cultures. Moreover, it was found that females were similarly affected, the ovaries remaining vestigial.

It was established that this sterility of the progeny was associated with particular female parents and was independent of the male parent. In order to determine the hereditary basis of the condition, brothers of grandchildless females were crossed with unrelated females and the progeny inbred. After two generations the sterile families reappeared, thus showing that their cause could be carried by males. Crosses were also made between brothers and sisters of 'sterile-progeny' females, and some the the female progeny were found to show the condition by having sterile offspring. In this way the sterility was followed through more than twenty generations of flies. Grandchildless females formed a minority of the females in each generation.

On the evidence of the crossing experiments, it was postulated that the sterility was due to a Mendelian recessive gene called *gs*. The sisters with fertile offspring, by which the *gs* stock was maintained, were assumed to be heterozygous normals (+/*gs*)*, but it was impossible to determine whether the brothers with which these were crossed in the inbreeding experiment were heterozygous (+/*gs*) or homozygous (*gs*/*gs*). On the hypothesis just mentioned of a Mendelian recessive factor determining the grandchildless condition, in each generation following brother-sister mating, either a 3:1 or a 1:1 ratio of 'fertile-progeny' to 'sterile-progeny' females is expected, but the individual families were too small to distinguish these ratios. Taking Spurway's total data for fifteen consecutive generations (F_8 to F_{22} inclusive) comprising 23 brother-sister matings, the female progeny consisted of 309 with fertile offspring, 90 with sterile offspring and 112 themselves sterile. Since grandchildless females are less fertile than normal, it is probable that the 112 sterile females include many *gs*/*gs* females. With any reasonable distributions of the 112 between the other two classes, the ratio of the latter lies between 3:1 and 1:1, and hence is in agreement with the hypothesis.

* The + sign is used for the normal allele.

It thus appears that a Mendelian recessive factor, when homozygous, leads to the production of some substance in the ovary of female flies. This substance, or some other effect of the gene, may lower their fertility somewhat, but its main effect is on the progeny. It is presumably carried via the cytoplasm of the egg to the next generation, where it leads to a failure of development of the ovaries and testes. The abnormality cannot be detected before the last instar in male larvae, or the mid-pupal stage in females, and is most readily observed in the mature insects. This represents one of the most extreme instances known of the delayed effect of a Mendelian gene.

§ 5.8 Rhoades' experiments with iojap *Zea*

An instructive comparison can be drawn between the inheritance of grandchildless *Drosophila subobscura* and that of a particular kind of leaf-striping in *Zea mays* called 'iojap'. The name 'iojap' is abbreviated from 'Iowa', as the source of the maize strain, and 'japonica' as the name of a similar striped variety. Jenkins (1924) described experiments which established that the striped leaves found in iojap plants are due to a recessive Mendelian gene. Maize is usually cross-fertilised. Certain normal green plants when self-pollinated were found to give progenies segregating for iojap striping. Omitting one family where the stripes were very narrow and hard to recognise, Jenkins' total data for these segregating progenies were 2498 green and 782 iojap (including 12 white plants) (Fig. 5.6(*a*)) (Expected frequencies on 3:1 hypothesis: 2460 and 820, deviations 38, $\chi^2 = 2{\cdot}3$, $n = 1$, $P > 0{\cdot}1$.) Furthermore, 26 of these 2498 green plants, on self-pollination, were found to consist of 9 which bred true and 17 which again

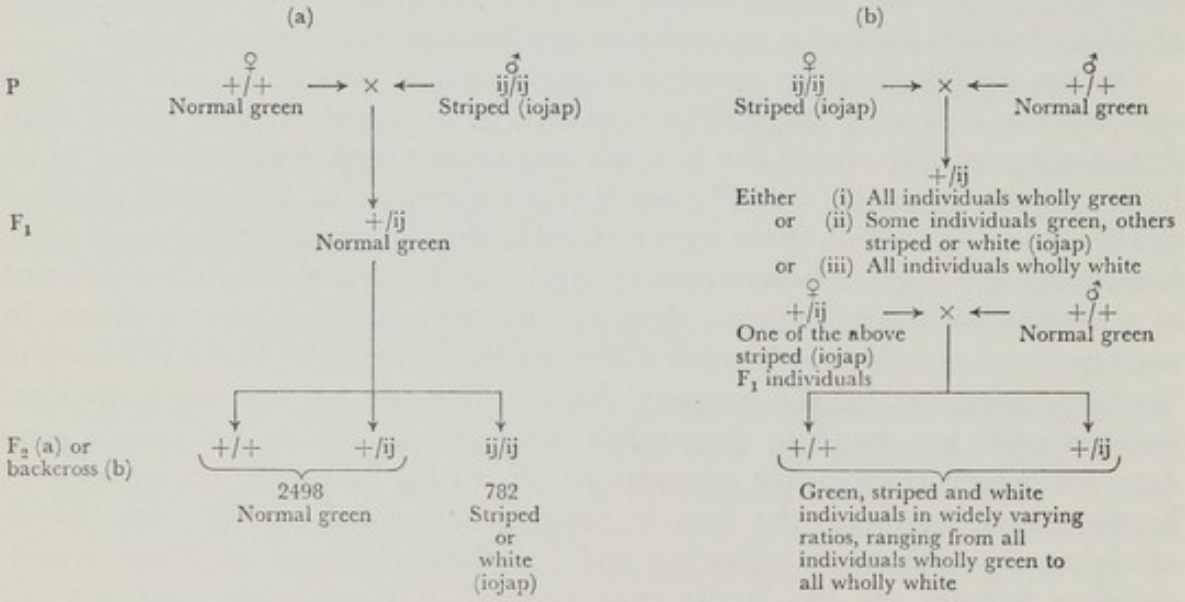


FIGURE 5.6 Inheritance of iojap character in *Zea mays*.
(*a*) Mendelian inheritance when iojap plants used as male parents (from Jenkins, 1924).
(*b*) Non-Mendelian inheritance when iojap plants used as female parents (from Rhoades, 1943).

segregated for iojap, in good agreement with the 1:2 ratio of homozygotes to heterozygotes which Mendel would have expected. However, an unexpected result was obtained when female inflorescences of iojap plants (obtained as above) were pollinated from a pure-breeding normal green plant. The resulting F_1 plants often had striped leaves of typical iojap appearance. Rhoades (1943) showed that so long as iojap plants are used as female parents, the inheritance of the condition is similar to that of the *albomaculata* variety of *Mirabilis jalapa* described by Correns (1909) and discussed in § 2.7. Thus, widely varying ratios of green, white and striped progeny were obtained in this way from iojap plants, and this result was found irrespective of the male parent used (Fig. 5.6(*b*)).

Rhoades proposed the following hypothesis to account for these remarkable results. The nuclear gene, *ij*, when homozygous (*ij*/*ij*) causes striping of the leaves, but this striped condition, once it has been initiated, is then inherited through the cytoplasm. Cytoplasm is handed down only through the egg-cell and not through the pollen, and so the iojap condition shows maternal inheritance. Rhoades confirmed this hypothesis by crossing the F_1 striped plants as female parent with an unrelated normal green plant as male parent (see Fig. 5.6(*b*)). The F_1 striped plants would be heterozygous for the iojap gene (+/*ij*), the other parent would be homozygous for the normal allele (+/+), and so the progeny from this cross would consist of equal numbers of individuals homozygous for the normal allele (+/+) and heterozygous (+/*ij*). Nevertheless, for some ears the progeny consisted entirely of white seedlings, half of which by the above reasoning must not have possessed the iojap gene at all. From other ears, there were green, variegated and white progeny in diverse ratios, and here again many of the iojap plants must have lacked the iojap gene.

The essential difference between the inheritance of grandchildless *Drosophila* and iojap *Zea* is that in the former the cytoplasmic modification initiated by the nuclear gene is passed only to the immediate progeny and no further, whereas in the latter it appears to be capable of indefinite passage from generation to generation. This distinction constitutes the essential difference between Mendelian and non-Mendelian inheritance. It can of course be argued that in grandchildless *Drosophila* there is no further opportunity for cytoplasmic passage beyond the immediate progeny, on account of the sterility induced. The initiation of iojap *Zea* shows Mendelian heredity, but once it has been initiated, the inheritance is non-Mendelian. The ability to be handed down indefinitely, independently of the nuclear constitution, implies that whatever is responsible for non-Mendelian inheritance must possess the ability to reproduce itself in the cytoplasm. The nature of such reproducing structures is discussed later in this chapter and in § 18.11.

§ 5.9 Baur's experiments with *Pelargonium*

Almost simultaneously with Correns' (1909) discovery of the non-Mendelian inheritance of the *albomaculata* character in *Mirabilis jalapa* (see Chapter 2), Baur (1909) reported an example in *Pelargonium zonale*

(Geranium) which differed in an important respect from that in *Mirabilis*. Baur's example also concerned leaf-variegation, but he found that in *Pelargonium* reciprocal crosses gave similar results. It will be recalled that in 'albomaculata' *Mirabilis* (and also in 'iojap' *Zea*) such crosses give different results. Plants of a strain of *Pelargonium* with white-margined leaves when self-pollinated or inter-crossed gave wholly white progeny, which necessarily died young. Baur found that the sub-epidermal layer in white-margined plants was white. Since this is the layer which gives rise to the reproductive cells, the wholly white progeny could be explained. However, when the white-margined plants were crossed with normal green plants, the progeny consisted of green plants, white-margined plants and wholly white plants in widely-varying ratios, and this was true which ever direction the cross was made. Baur concluded that in *Pelargonium* cytoplasm is inherited from the male as well as the female parent. Thus the difference between reciprocal crosses, which in the *Mirabilis* and *Zea* examples was quoted as one of the distinctions from Mendelian heredity, cannot be maintained as an invariable feature of non-Mendelian heredity. The most striking feature of the latter kind of inheritance is the failure of the character-difference to segregate in a regular and predictable way at the time of formation of the reproductive cells.

§ 5.10 Renner's experiments with *Oenothera*

Further examples of non-Mendelian heredity of the *Pelargonium* type were found by Renner (1924) in *Oenothera* (Evening Primrose) hybrids. Thus, he found that when *O. hookeri* was crossed as female parent with the not very closely related *O. muricata* as male parent, the hybrids had yellow leaves and died. Plants of the same nuclear constitution obtained from the reciprocal cross* had green leaves with yellow flecks. He postulated that these yellow flecks represented cytoplasm contributed by the male parent (*O. hookeri*) and that in the presence of the hybrid nucleus, the *O. hookeri* cytoplasm became defective, giving the yellow colour. From self-pollination of flowers on wholly yellow shoots on the flecked plants, Renner was able to show that normal green progeny were recovered whenever the cytoplasm was restored to an *O. hookeri* nucleus. This result confirmed his hypothesis that the yellow colour was a consequence of lack of harmony between *O. hookeri* cytoplasm and hybrid nucleus, and showed that the change was reversible.

§ 5.11 Hypotheses to explain non-Mendelian inheritance of plastid characters

From the discussion of non-Mendelian heredity, both in this chapter and in Chapter 2, it is apparent that this type of inheritance is best explained as due to the transmission of some material in the cytoplasm. All the examples mentioned have referred to abnormalities of the green pigmentation of the

* To obtain such plants is more difficult than might be supposed, owing to the occurrence in *O. muricata* of an abnormal hereditary mechanism (see § 9.2).

leaves. The chlorophyll occurs in particles in the cytoplasm called plastids. The simplest hypothesis to account for the non-Mendelian inheritance of chlorophyll-pigmentation defects is therefore to postulate that plastids are autonomous self-replicating structures and that they may occur in abnormal yellow or colourless forms. This hypothesis was first proposed by Baur (1909) to account for his results with *Pelargonium*, and is supported by several pieces of evidence, notably that the white areas in variegated plants are found to have minute colourless plastids visibly different from the normal green ones characteristic of the green areas. However, it does not follow from this that the plastids are necessarily themselves the hereditary determinants. Indeed, the following observations made by Correns (1909) with 'albomaculata' *Mirabilis jalapa* suggest that cytoplasmic constituents other than the plastids are responsible. First, at the boundary between green and white areas in variegated plants, a gradation in plastid colour was observed from cell to cell, suggesting diffusion from the white areas of some substance which interferes with normal plastid development. Secondly, cells were rarely found to contain a mixture of two different kinds of plastids such as on Baur's hypothesis would be expected to occur frequently, particularly in the boundary zone between green and white areas. Thirdly, there are insufficient cell-divisions during development for the chance segregation of two types of plastids into different cells to occur often, yet green and white areas are constantly being initiated. Similar observations to these have been made in a number of other instances of non-Mendelian inheritance of variegation, for example by Rhoades (1947) with iojap *Zea*.

If the cytoplasmic hereditary determinants are not the plastids, what is their nature? In attempting to answer this question, an observation by Anderson (1923) is important. He was the first to discover non-Mendelian inheritance of leaf-striping in *Zea mays*, and in studying the progeny of such a striped plant used as female parent, he took care to record the position of each grain on the ear. He found that all the grains in one area of the ear produced green plants, and all those in another area produced white plants that died, while striped plants tended to come from grains forming a transitional zone between the 'green' and 'white' areas (see Fig. 5.7). These areas extended longitudinally up the ear and corresponded with the cell-multiplication during development, just as in the vegetative parts the striping reflects the way in which the leaf develops. It is evident that, in sexual reproduction as well as in leaf growth, green cells tend to give green, and white to give white. This behaviour suggests that the hereditary determinants do not readily diffuse from one cell to another, or in other words are particles rather than a fluid. It should be noted that the gradation in plastid colour at the boundary of green areas, referred to in the last paragraph, need not imply that the hereditary determinant is fluid but merely that its effects can diffuse from one cell to another. Similar gradations in plastid colour often occur when variegation shows Mendelian inheritance, and then one concludes that the hereditary determinant resides in the cell nucleus, and hence cannot be directly responsible for the gradation in colour in the cytoplasm.

FIGURE 5.7 Reconstruction (×0·5) of an ear on a striped plant of *Zea mays* studied by Anderson (1923). White (unshaded) grains gave albino seedlings, cross-hatched grains gave striped seedlings, and black grains gave normal green seedlings. All the grains on the ear looked alike. Almost all those on the rear side of the ear gave green seedlings.

This hypothesis of particulate cytoplasmic hereditary determinants, capable of replication and indefinite passage from generation to generation, appears to be consistent with the data on non-Mendelian inheritance of variations in the green pigmentation of plants. However, these hypothetical particles have not yet been identified with specific cytoplasmic structures. Certain of the data, notably those of Baur for *Pelargonium* and of Renner for *Oenothera*, are consistent with the equating of these particles with the plastids, but this equation lacks confirmation.

§ 5.12 Further examples of cytoplasmic inheritance

Cytoplasmic inheritance in green plants is not confined to plastid characters. Michaelis (1954) studied cytoplasmic inheritance in *Epilobium* (Willow-herb) by making reciprocal crosses between different species and

varieties, and then pollinating the progeny with the male parent, and repeating this in each successive generation. On the assumption that no cytoplasm is inherited from the pollen, plants will be obtained within a few generations which have essentially the nuclear constitution of one parent (that used as male in each generation), while the cytoplasm has all been derived from the other parent. Michaelis found that *E. luteum* as female crossed for 24 successive generations with *E. hirsutum* as male gave male-sterile plants resembling *E. hirsutum*. The reciprocal cross could not be continued for more than 3 generations of back-crossing because of sterility or inviability. Within the one species, *E. hirsutum*, similar back-crossing between strains from different parts of Europe also gave evidence of persistent cytoplasmic differences: such characters as male-sterility, stunted growth, or deformed flowers distinguished the back-cross progeny from their male parent. It is evident that some features of *Epilobium* cytoplasm are autonomous and persist, apparently indefinitely, in the presence of an alien nucleus.

Some cytoplasmically-inherited variants, for example, the killer character in *Paramecium aurelia*, and carbon dioxide sensitivity in *Drosophila melanogaster*, appear to be due to infection by a virus (cf. review in Jinks, 1964).

§ 5.13 The heterokaryon test

Many examples of non-Mendelian inheritance are known in Fungi. For this group of organisms, Jinks (1954) has devised a test for the occurrence of cytoplasmic variants, based on the phenomenon of heterokaryosis described in § 5.4. A mutant such as the white conidial character in *Aspergillus nidulans* is known to differ from the normal green conidial form by a nuclear gene, and a heterokaryon of the two produces conidia of each colour. These conidia are uninucleate, and so the colonies derived from them will be homokaryotic. If another character-difference also distinguishes the strains which give rise to the heterokaryon, these characters may reappear only in the parental combinations in the daughter homokaryons. It is then inferred that the second character-difference is also determined by the nucleus. However, it has been found that when a white sexually-fertile and a green sexually-sterile strain form a heterokaryon, the daughter homokaryons may all be sexually fertile whether white or green. It is then inferred that the sexual sterility of the green parent is of cytoplasmic origin. In a particular strain of *A. nidulans* which had been treated with ultraviolet light, a variant with red mycelium continually reappears in a proportion of the colonies derived from the conidia, even when these conidia are obtained from colonies of normal appearance. This is comparable to the origin of cytoplasmically inherited petite yeast described in § 5.15. Arlett, Grindle and Jinks (1962) isolated single conidia from a heterokaryon between this strain and a strain with white conidia. They found that recombination had occurred and the red variant now arose in some isolates of the white-conidial strain, as well as in some of those with normal green conidia. It was evident that the red variant was cytoplasmically inherited, since there is normally no recombination between nuclear factors in a heterokaryon.

§ 5.14 Specific agents which induce cytoplasmic variants

One of the characteristic features of cytoplasmic inheritance is the specific induction of variants by certain agents. This is in contrast to the production of Mendelian mutants, where mutagens produce a whole range of kinds of mutants, not one specific one (see Chapter 9). Agents known to cause specific cytoplasmic variants include acridine dyes, streptomycin, and high temperatures during growth. Treatment with streptomycin (Provasoli, Hutner and Schatz, 1948) or growth for 5 or 6 days at 34°C (Pringsheim and Pringsheim, 1952) can cause a permanent loss of the plastids in *Euglena gracilis*, which then becomes a colourless saprophytic organism, formerly regarded as a distinct species and called *Astasia longa*. Sager (1962) discovered that streptomycin caused cytoplasmic mutation in *Chlamydomonas reinhardi*. Such mutants are transmitted usually by one parent only, exceptionally by both. From study of the progeny of such exceptional zygotes, Sager and Ramanis (1963) found that segregation of two cytoplasmic differences (streptomycin dependence (*sd*) and sensitivity (*ss*), and acetate requirement (ac^-) and independence (ac^+)) occurred independently, the acetate character usually at an earlier stage of development than the streptomycin character.

§ 5.15 Ephrussi's experiments with yeast

One of the clearest examples of non-Mendelian heredity in Fungi concerns 'petite colonie' in *Saccharomyces cerevisiae* (Baker's Yeast) studied by Ephrussi and colleagues (see Ephrussi, 1953). When individual yeast cells are spread on culture medium, they soon grow to form small circular colonies containing many cells. Ephrussi observed that one or two out of every thousand such colonies were only about one-third or one-half the diameter of the remainder. Cells from one of the normal large colonies, when again spread on culture medium produced a further small proportion of 'petite' colonies, and this happened time after time. On the other hand, the cells from the small colonies bred true and gave only petites. Biochemical studies established that the slow growth of the petite strain of yeast was due to the loss of the ability to respire aerobically. In the absence of oxygen, the growth rate of large colonies was slowed to that of petites. Furthermore, the lack of aerobic respiration in the petite strain was found to be associated with the absence of a number of respiratory enzymes. These enzymes occur in particles in the cytoplasm called mitochondria. Ephrussi and associates discovered that in the presence of acridine dyes, such as acriflavine or euflavine, all the newly-formed daughter-cells in a yeast colony, if isolated, gave rise to petite colonies. This change from normal to petite, with its associated loss of respiratory enzymes, persisted indefinitely after the removal of the dye. When the petite strain, whether arisen spontaneously or induced by acridines, was crossed with a normal strain, the progeny did not show the petite character, and it did not reappear in subsequent generations apart from the low percentage of petite colonies which were always arising. Other character differences, when present, showed normal Mendelian inheritance, with precise ratios.

A number of similar variants with deficiencies in several respiratory enzymes are also known in *Neurospora crassa* (see Fincham and Day, 1965, and Jinks, 1964).

To account for his results, Ephrussi put forward the hypothesis that the petite strain differs from the normal in the loss of cytoplasmic particles postulated to be necessary for the synthesis of the respiratory enzymes. If these hypothetical particles are distributed at random between the parent cell and the daughter-cell budded off from it, and if it is further assumed that there are normally only about 10 of the particles per cell, then about one per thousand daughter-cells will contain none of the particles and will give rise to a petite colony. This would be in agreement with observation. The acridine dyes are assumed to destroy the particles or to prevent them from multiplying. When a petite strain is crossed with a normal one, the petite character is lost because some of the cytoplasm of the normal parent is passed to all the progeny, which therefore receive the postulated particles. Thus it can be seen that this hypothesis will account satisfactorily for all the observations.

It will be noticed that this hypothesis resembles that proposed to explain non-Mendelian inheritance of green pigment variants in flowering plants discussed above. Both theories invoke particulate cytoplasmic determinants. The resemblance may extend further, for the white areas in variegated plants may be composed of cells which have lost the particles necessary for the development of normal plastids, just as the petite yeast cells are thought to have lost particles necessary for the development of normal mitochondria. Moreover, just as plastid variants may show Mendelian inheritance (for example, yellow *versus* green seeds in *Pisum*), Ephrussi found a dwarf strain of yeast which showed Mendelian inheritance. This strain lacked the ability to respire aerobically, just like the non-Mendelian petite strain already described. It appears that particulate hereditary factors responsible for fundamental cell phenomena such as photosynthesis and respiration occur in both nucleus and cytoplasm.

§ 5.16 Conclusion

Evidence has been brought together in this chapter for the belief that Mendelian heredity, with its regular segregation of character-differences to give simple ratios of different kinds of progeny, and with its identical results from reciprocal crosses, is brought about by particulate determinants in the cell-nucleus, although their effects may sometimes be delayed. Conversely, non-Mendelian heredity, characterized by a lack of regular segregation of character-differences, by variable progeny ratios, and usually by maternal inheritance, appears to be determined by cytoplasmic particles. Whereas the identity of the nuclear particles is well-established and will be discussed in subsequent chapters, that of the cytoplasmic particles is less certain (see 18.11). The relative importance of nucleus and cytoplasm in heredity is discussed in Chapter 18.

6. *The chromosome theory of Mendelian inheritance*

§ 6.1 Chromosomes

Thread-like structures which readily stain with many dyes had long been noticed at nuclear divisions before careful and detailed observations, notably by Flemming (1882) with *Salamandra maculosa* (Spotted Salamander), Strasburger (1882) with various plants, and van Beneden (1883) with *Parascaris equorum*) (*Ascaris megalocephala*, Horse Thread-worm), established the essential features of the division process. Flemming (1879) introduced the term *chromatin* for the deeply staining material of nuclei, and (1882) *mitosis* for the process of division. Various nouns such as 'threads', 'loops', 'segments' or 'elements', qualified by such adjectives as 'primary', 'nuclear' or 'chromatic' had been used for the structures seen during mitosis, until Waldeyer (1888), in reviewing what was known about nuclear division, proposed the name *chromosome* for them, because 'they are so important', adding that if this name 'is practically applicable it will become familiar, otherwise it will soon sink into oblivion' (English translation, p. 181).

§ 6.2 Mitosis

The key observations on mitosis were made by Flemming (1882) and van Beneden (1883). Flemming discovered in the salamander larva that the chromosomes divided longitudinally during mitosis, and van Beneden observed in *Parascaris equorum* that the two daughter-chromosomes derived from one original chromosome were exactly alike down to the smallest detail, and moreover that they separated and passed one to each daughter-nucleus. It thus became apparent that mitosis was essentially a mechanism for distributing the halves of longitudinally-split chromosomes equally to two daughter-nuclei. The longitudinal splitting was seen to be of such paramount importance that Strasburger (1884) coined the terms *prophases* and *metaphases* to refer to the stages of mitosis before (*pro-*) and after (*meta-*) the chromosomes had separated into daughter-halves, with *anaphases* for the stages when the daughter groups of chromosomes were going back (*ana-*) to the appearance of the original parent-nuclei. Subsequently, more careful observation revealed that the chromosomes divide earlier than was originally supposed, and indeed it is now generally accepted that the threads are double from the earliest stage of mitosis at which they can be seen. The term

telophase was added by Heidenhain (1894) for the final stages of mitosis, with particular reference to human leucocytes, and about 1905 Strasburger began to use his terms for earlier stages of mitosis than he had originally. These revised usages are those now universally adopted.

The so-called resting-nucleus, prior to the beginning of mitosis, is a more or less spherical structure, bounded by the nuclear membrane and containing the nuclear sap, one or more *nucleoli*, and the chromatin. The nuclear membrane has been shown from electron micrographs to be a double membrane and to contain pores about 40 mμ in diameter (see Pl. 4(*a*)). When a cell is fixed and treated with dyes, the nucleoli and the chromatin usually stain deeply whereas the nuclear sap remains unstained. The nucleoli are small spherical structures. Each is attached to a *nucleolar organizer* on a particular chromosome. A possible function for the nucleoli is discussed in §18.7. The chromatin gives the appearance of a network of threads filling the nucleus, but even at the time of Flemming and Strasburger's pioneer observations, the existence of a physical reticulum was questioned, and it is now generally agreed that the chromosomes retain their identity through the resting-stage, although invisible. The fact that there appeared to be characteristic chromosome numbers for each species argued in favour of the continuity of the chromosomes. Moreover, Boveri (1888) showed for the first few mitoses in the fertilised egg of *Parascaris equorum* that the chromosomes re-appeared at prophase in the position they had occupied at the preceding telophase. In recent years this has been clearly shown by phase-contrast photography of living nuclei, such as Bajer's ciné films of mitosis in the endosperm of *Haemanthus katherinae* and *Clivia cytranthiflora* (see Bajer, 1957–58). The reticular appearance of nuclei in the resting-stage is thought to be an optical effect associated with the existence of the chromosomes in the form of threads so fine as individually to be beyond the resolving-power of the microscope.

Mitosis is initiated (*prophase*) by the chromosomes gradually becoming more distinct. It was formerly thought that all the chromosomes might be joined end-to-end at this stage but here also it is now generally agreed that they are not joined. The early studies were made by fixing the plant or animal material, embedding it in wax, and then cutting a series of thin sections with a microtome. The thickness of these sections was commonly of the same order as the diameter of the nucleus, so that nuclei were frequently cut open. If free ends of chromosomes were seen, it was often thought that these were artefacts due to the cutting. Nowadays, squash techniques rather than sections are usually employed.

The chromosomes become thicker and shorter during prophase through spiral contraction. The direction of coiling is variable. As already indicated, the prophase chromosomes are seen to be double structures, each having divided longitudinally into two identical halves called *chromatids*. The two halves, however, remain closely associated throughout their length (see Fig. 6.1(*b*) and Pl. 1(*a*)), and are twisted round one another. In late prophase this relational coiling disappears. The uncoiling is well seen in Bajer's film. The nucleoli diminish in size and ultimately disappear, and shortly afterwards

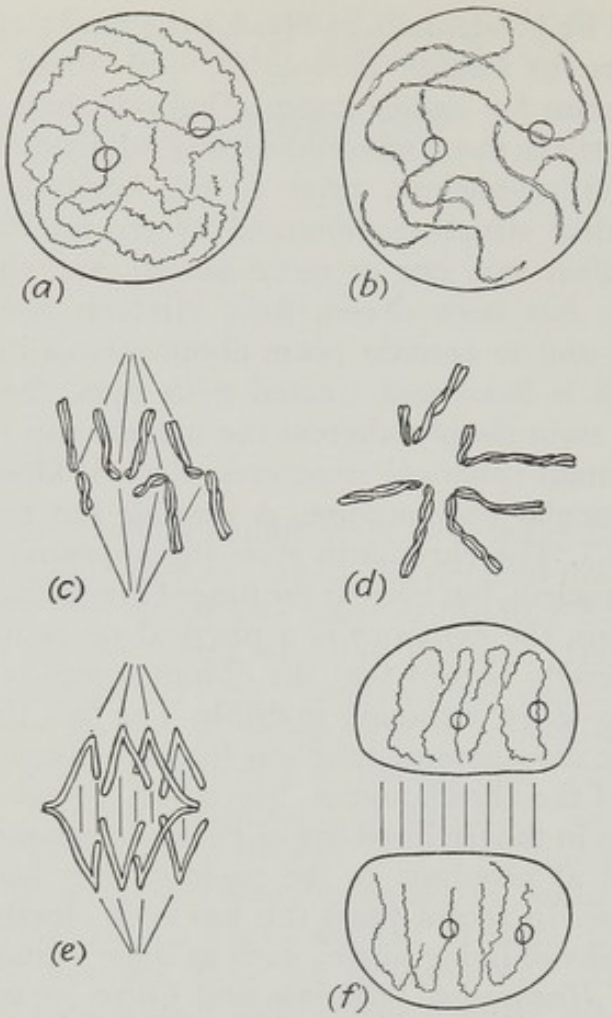


FIGURE 6.1 Stages of mitosis in an organism with two pairs of chromosomes.
(*a*) Early prophase. The small circles represent nucleoli.
(*b*) Late prophase.
(*c*) Side view of metaphase.
(*d*) Polar view of metaphase.
(*e*) Side view of anaphase.
(*f*) Telophase.

the nuclear membrane breaks down, thereby releasing the chromosomes in the cytoplasm of the cell. It is customary nowadays to identify this relatively abrupt breakdown of the membrane with the end of prophase.

At the beginning of the succeeding stage, *metaphase*, a series of faint lines, which do not stain with dyes, become visible forming a spindle-shaped structure in the cytoplasm of the cell. In animals the spindle has well-defined poles—star-like structures from which the faint lines radiate. In plants, the lines fade away before the poles are reached. The chromosomes, now relatively short and thick, each with its two chromatids still closely paired (Pl. 1(*b*)), become arranged in the equatorial plane across the middle of the spindle. Each chromosome is usually bent at a sharp angle at one point to give

a shape like the letter *V* as seen from the poles of the spindle. The angles of the *V*'s all point towards the centre of the equatorial plate. The tip of the angle in each chromosome coincides with a non-staining constriction called the *centromere*. It is the centromeres which appear to be responsible for the chromosome movement on to the metaphase plate. The spindle-fibres, as the fine non-staining lines in the cytoplasm are called, pass through individual centromeres. The chromosome arms, if long, usually point towards one or other pole (see Fig. 6.1(*c*)). In a few organisms, for example, species of *Luzula* (Wood-rush) and certain hemipterous insects, chromosome movement appears to be determined through a generalized property of the chromosomes rather than through a discrete entity, the centromere. Such organisms are said to have diffuse centromeres. Their chromosomes do not show the characteristic *V* or *L* shape of normal chromosomes at metaphase.

Anaphase is said to begin when the daughter-centromeres start to separate, each moving along a spindle-fibre in opposite directions towards the poles. The daughter-chromosomes thus separate first at the angle between the two arms (Pl. 1(*c*)). The last point of separation is the tip of the longer arm (Fig. 6.1(*e*)). Mazia (1955) has isolated the spindle from fertilised eggs of the echinoderm *Strongylocentrotus purpuratus* undergoing mitosis, by removal of the rest of the cytoplasm with the natural steroid detergent digitonin. Chemical analysis showed that the spindle fibres are composed largely of protein, and the amino-acid composition of this protein was found to resemble that of the protein of animal muscles. It was therefore suggested that the shortening of the spindle fibres, which occurs in the anaphase movement, has features in common with muscular contraction. A second factor in the anaphase separation of the daughter-chromosomes is the elongation of the middle region or body of the spindle lying between the two groups of chromosomes. Ris (1949) showed that this elongation is inhibited by chloral hydrate, whereas the spindle fibre contraction is not.

Telophase is said to be initiated when nuclear membranes form round each group of chromosomes (Fig. 6.1(*f*)). Nucleoli re-appear within each daughter-nucleus, and the chromosomes, each now composed of a single chromatid, gradually become longer and thinner until the resting-stage or *interphase* is reached, when they can no longer be clearly seen.

From this brief outline of mitosis it is evident that the duplication of the chromosomes takes place during the resting-stage. This has been confirmed from isotope labelling and other experiments (see Chapter 11).

§ 6.3 Meiosis

An observation of great significance was made by van Beneden (1883) in his studies of *Parascaris equorum*. He noticed that the egg and sperm nuclei each contained two chromosomes, whereas at the mitoses in the fertilised egg four chromosomes were visible. He thus established that the gametes each contribute the *haploid* number of chromosomes to the zygote, which thus contains the *diploid* number in its nucleus. Weismann (1887) predicted that there must be a special nuclear division, repeated in every generation,

at which the chromosome number is reduced again to half the number contained in the parent nucleus. It was already known that the nuclear divisions just before the maturation of sperm and eggs in animals, and before the formation of pollen-grains and embryo-sacs in flowering plants, were abnormal. Two nuclear divisions were found to occur in quick succession. Flemming (1887) called them *heterotype* and *homotype*, respectively, since the first was the more abnormal of the two. Strasburger (1888) observed in the pollen-mother-cells and in the embryo-sac-mother-cells of various flowering plants that at late prophase of the first of these divisions, when the chromosomes first become clearly visible, the haploid number is present. Whether these were individual chromosomes, or pairs of chromosomes, and if the latter, whether the chromosomes of each pair were associated laterally or terminally, were long disputed, but it soon became evident that Weismann's predicted reduction was occurring during these divisions, since at the preceding mitosis the diploid number of chromosomes was visible. Farmer and Moore (1905) gave the name *meiosis*, meaning reduction, to the whole process of two successive nuclear divisions. Grégoire (1904) had already suggested that the two divisions should be distinguished by the Roman numerals I and II.

§ 6.4 Von Winiwarter's observations on meiosis in rabbits

As already indicated, much difference of opinion persisted for many years about the precise sequence of events during the early stages of meiosis I. This was partly owing to the difficulty of distinguishing younger and older cells in fixed preparations. However, von Winiwarter (1900) discovered that the ovary of *Oryctolagus cuniculus* (Rabbit) provided ideal material, since meiosis was found to extend over a long period of time. By examining the stage reached in young rabbits of known age, an unequivocal seriation was achieved.

The majority of the nuclei in the ovaries of a half-day old animal were found to contain chromosomes in the form of exceedingly fine single threads (Fig. 6.2(*a*) and Pl. 2(*b*)). Cells at this stage he described as having *leptotene* nuclei, meaning that the chromosomes were in the form of a 'slender ribbon'. Later work showed that the leptotene chromosomes have a highly characteristic appearance resembling a string of beads of unequal size unequally strung. The granules are called *chromomeres*.

Nearer the centre of the ovaries of the half-day old rabbit, von Winiwarter found a few nuclei in which the chromosomes had associated in pairs side by side for part of their length, but remained independent elsewhere (Fig. 6.2(*b*) and Pl. 2(*c*)). These he called 'synaptene' nuclei, meaning 'uniting ribbons', but owing to confusion over the meaning of the term 'synapsis', the synonym *zygotene* was substituted by Grégoire (1907). In rabbits aged $1\frac{1}{2}$ and $2\frac{1}{2}$ days from birth, von Winiwarter found abundant zygotene nuclei, with leptotene nuclei also present but chiefly nearer the periphery of the ovaries.

In the inner parts of the ovaries of 4- and 5-day old rabbits he found nuclei in which the chromosomes were thicker than at the earlier stages, and

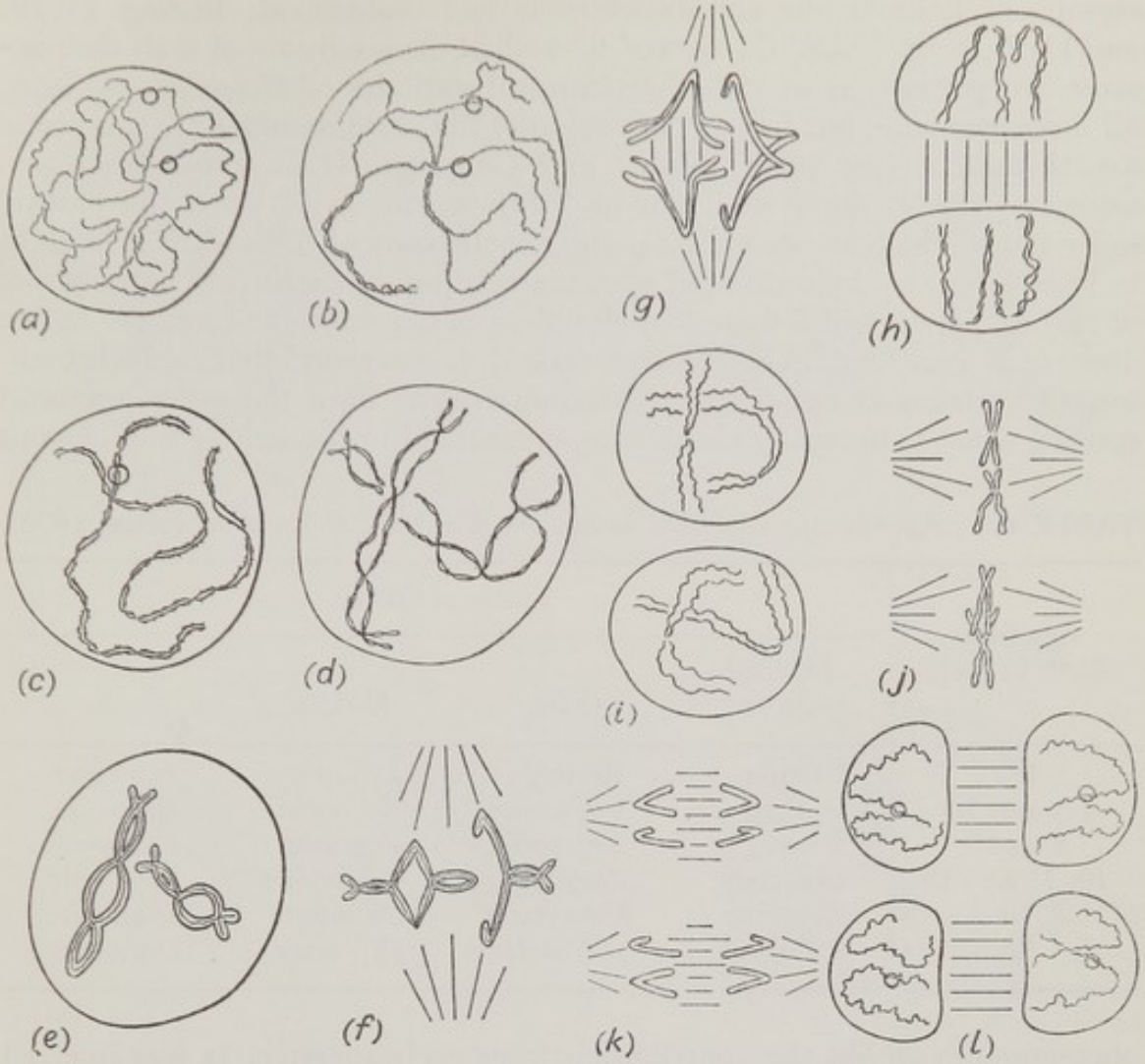


FIGURE 6.2 Stages of meiosis in an organism with two pairs of chromosomes.
(*a*) Leptotene stage. The small circles represent nucleoli.
(*b*) Zygotene stage.
(*c*) Pachytene stage.
(*d*) Diplotene stage.
(*e*) Diakinesis.
(*f*) Metaphase I (side view).
(*g*) Anaphase I (side view).
(*h*) Telophase I.
(*i*) Prophase II.
(*j*) Metaphase II.
(*k*) Anaphase II.
(*l*) Telophase II.

were associated in pairs throughout their length. To these he gave the name of nuclei with 'thick ribbons', or *pachytene* nuclei (see Fig. 6.2(*c*) and Pl. 2(*d*)). Outside these cells there was an extensive zone of cells with zygotene nuclei, and near the periphery of the ovary a few still at the leptotene stage. Wenrich (1916) observed in the grasshopper *Phrynotettix tschivavensis* (= *P. magnus*) that the chromosome pairing at the zygotene and pachytene stages was highly specific, with corresponding chromomeres closely associated with one another. The mechanism of this specific pairing of homologous

regions of homologous chromosomes is not understood. Belling (1929) and Darlington (1929*b*) discovered that when three editions of each chromosome are present, as in some horticultural varieties of *Hyacinthus orientalis*, all three associate, but in any one region of the chromosome only two are in actual contact (see §8.14). Riley and Chapman (1958) and Sears and Okamoto (1958) discovered that in *Triticum aestivum* (Wheat) the pairing behaviour of the chromosomes is under genetic control. This plant is thought to have arisen by hybridisation of ancestral species, each with a similar set of chromosomes, and followed by doubling of chromosome number. In the absence of a particular chromosome (no. 5), it was found that each chromosome associated at meiosis with its counterparts from the other ancestral species, whereas normally the pairing is confined to the strictly homologous

TABLE 6.1 Appearance of nuclei in ovaries of rabbits (Von Winiwarter, 1900).

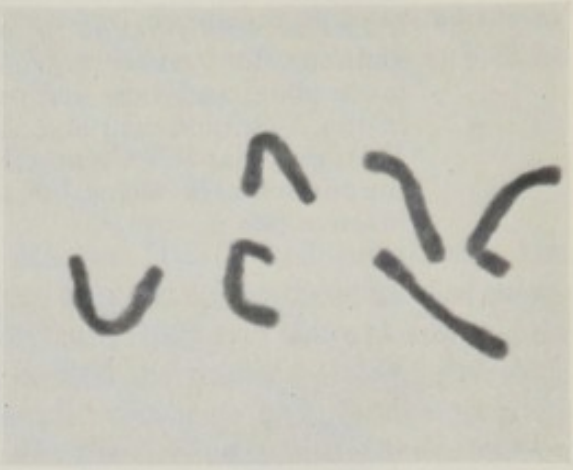
Age from Birth in Days	Tissue of Ovary			
	Extreme Outer	Outer	Middle	Inner
0·5	Resting	Resting	Leptotene	Zygotene
1·5 and 2·5	Resting	Leptotene	Zygotene	Zygotene
4 and 5	Resting	Leptotene	Zygotene	Pachytene
10, 11 and 12	Leptotene	Zygotene	Pachytene	Diplotene
18	Zygotene	Pachytene	Diplotene	Diplotene
28	Pachytene	Diplotene	Diplotene	Diplotene

chromosomes within the contribution from each ancestor. It was inferred that a specific gene in chromosome no. 5 determined the meiotic behaviour of all the chromosomes.

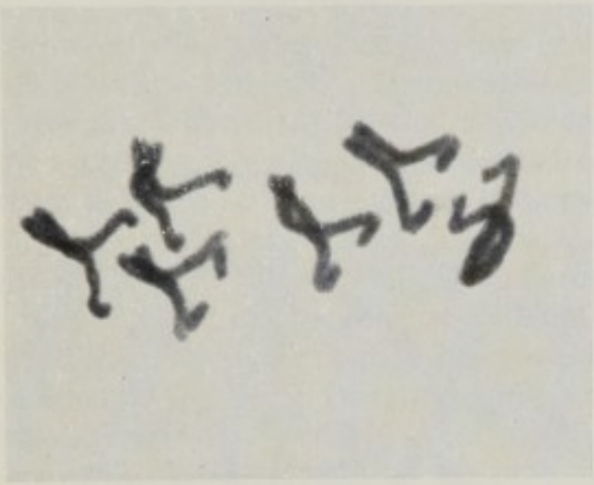
From rabbits aged 10, 11 and 12 days von Winiwarter found that the nuclei in the central parts of the ovaries contained chromosomes which were even shorter and thicker than the pachytene ones, and moreover had a very evident duality of structure, forming characteristic loops (see Fig. (6.2(*d*) and Pl. 2(*e*)). These nuclei he described as *diplotene*. The greater part of the ovaries of this age contained pachytene nuclei, with a peripheral zone at the zygotene and a few at the leptotene stage. An 18-day old rabbit had ovaries containing a thick zone of diplotene nuclei with many pachytene and a few zygotene nuclei near the outside, while at 28 days from birth there were no zygotene and only a few pachytene nuclei and the majority of the ovary cells showed nuclei at the diplotene stage. These results are summarised in Table 6.1, where the sequence leptotene–zygotene–pachytene–diplotene is clearly brought out. In the ovaries of vertebrate animals, meiosis commonly stops at the diplotene stage for a long period of time, so von Winiwarter was not able to follow meiosis beyond this stage in the young female rabbit. He observed the same stages as he had seen in the rabbit in the ovaries of a seven-month old human foetus.

PLATE 1 (*a*)–(*d*) Stages of mitosis seen in acetic-orcein squash preparations of root-tip cells of *Crocus balansae* (2n = 6). Magnification ca. ×750.
(*Courtesy of S. A. Henderson*)

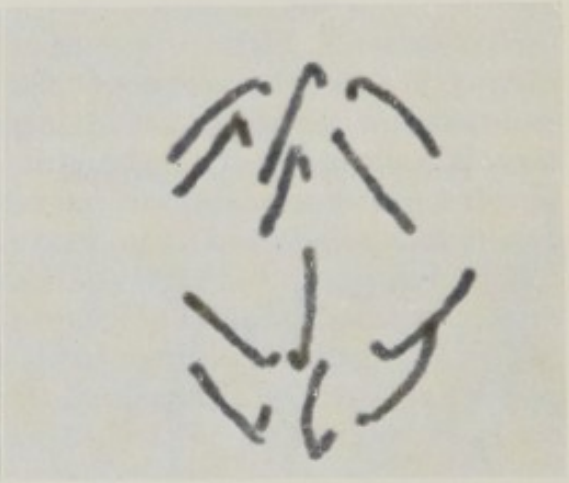
(*a*) *above* Mid-prophase



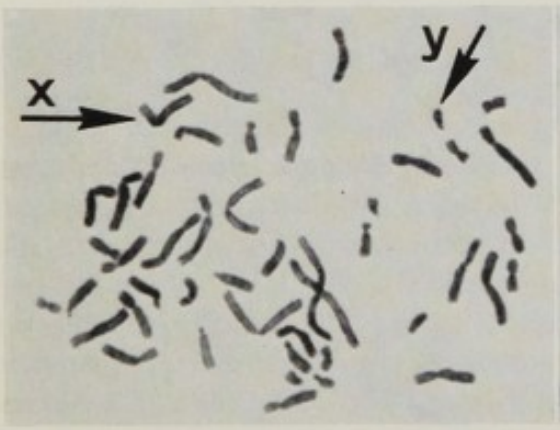
(*b*) *above* Metaphase



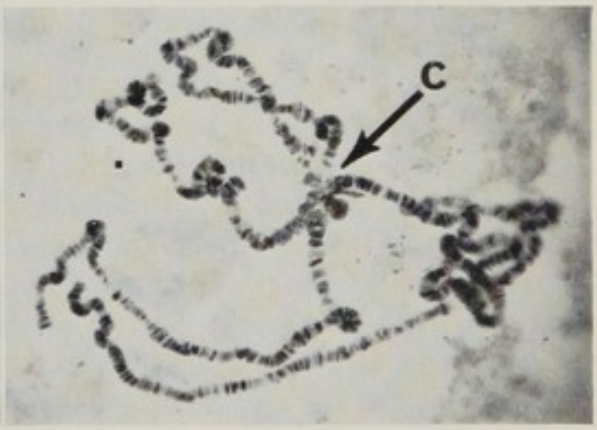
(*c*) Early anaphase



(*d*) Late anaphase

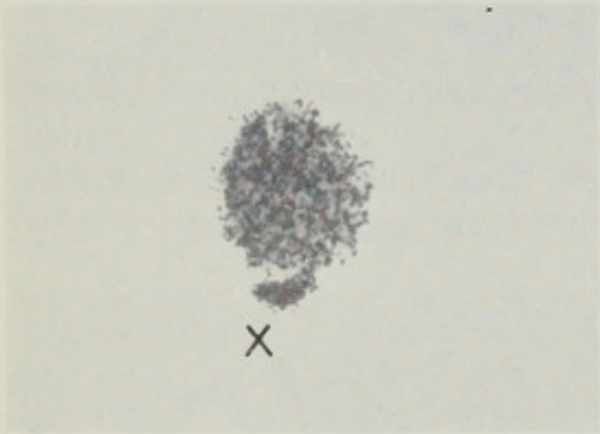


(*e*) Metaphase of mitosis in a leucocyte from peripheral blood of a human male (2n = 44 + *X* + *Y*). The material was treated with colchicine before fixation, and is a squash preparation stained with acetic-orcein. Magnification ca. ×400. *X* = probable *X*-chromosome. *Y* = probable *Y*-chromosome.
(*Courtesy of D. Briggs*)

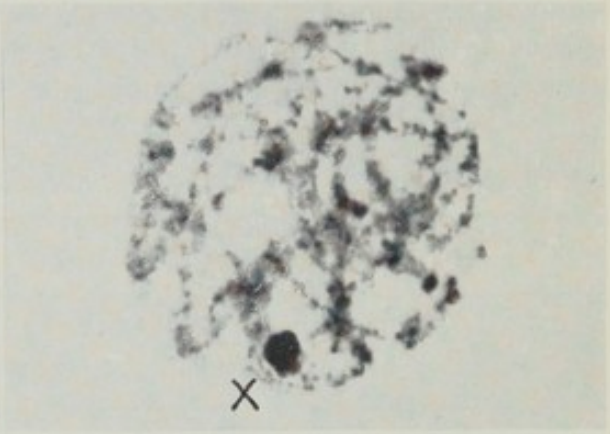


(*f*) A squash preparation of salivary gland chromosomes from a mature larva of *Drosophila melanogaster*, stained with acetic-orcein (cf. Fig. 9.1, p. 143) Magnification ca. ×400. *C* = Chromocentre.
(*Courtesy of D. Briggs*)

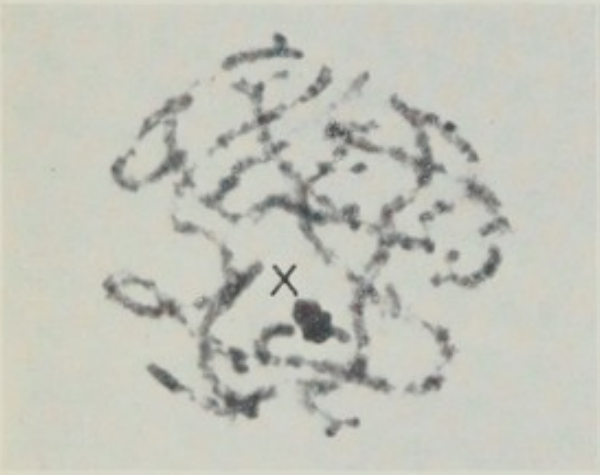
PLATE 2 The course of meiosis as seen in acetic-orcein squash preparations of spermatocytes of the locust *Schistocerca gregaria* ($2n = 22 + X$ in the male) (continued on Plate 3). Magnification ca. ×1000. X = X-chromosome. In (*a*) and (*f*), which are autoradiographs (4 days' exposure), the tissue was treated for 1 hour at 30°C with either 100 μC/0·2 ml of ^{3}H-thymidine (*a*) or 100 μC/0·1 ml of ^{3}H-uridine (*f*) before fixation. The grains reveal where uptake of the radioactive label has taken place, and show that preleptotene DNA synthesis (*a*) has occurred in the X-chromosome and the autosomal region of the nucleus (cf. p. 173), and that RNA synthesis at early diplotene (*f*) has taken place in the autosomal bivalents, but not in the X-chromosome which is evidently inactive (see p. 364). (*Courtesy of S. A. Henderson*)



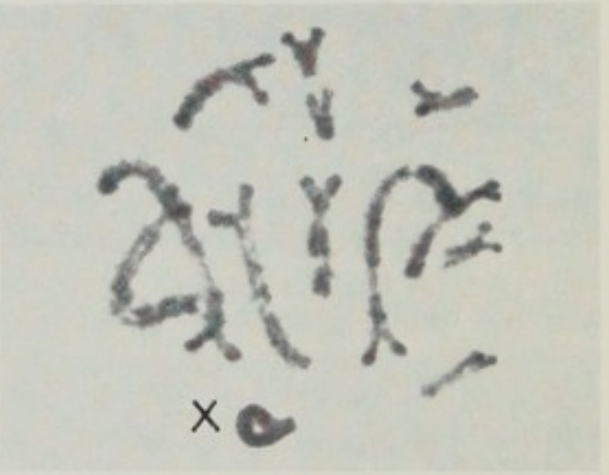
(*a*) Preleptotene (autoradiograph)



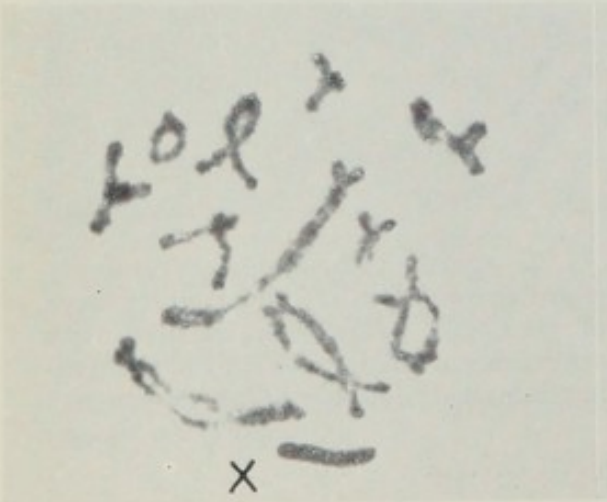
(*b*) Late leptotene



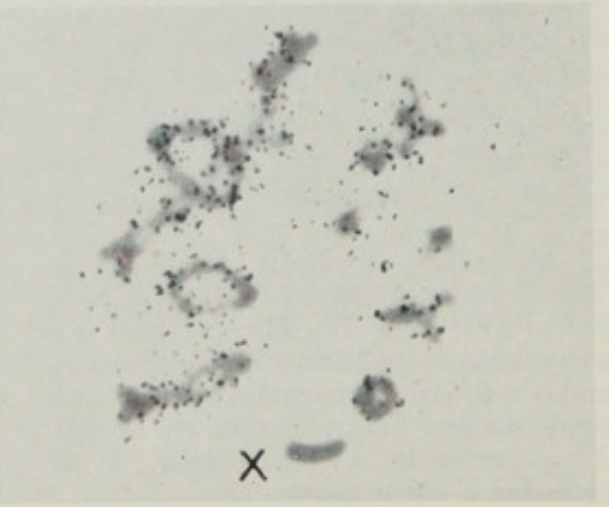
(*c*) Late zygotene



(*d*) Late pachytene



(*e*) Early diplotene



(*f*) Early diplotene (autoradiograph)

Dispute continued for a further 30 years after von Winiwarter's pioneer studies, before general agreement was reached that his observations were correct and applied throughout the plant and animal kingdoms wherever meiosis took place. The reasons for this long drawn out controversy will be discussed in Chapter 8.

§ 6.5 Diakinesis

Prior to von Winiwarter's studies, Häcker (1897) had subdivided the prophase of the first division of meiosis into two parts. For the second of these, when the chromosomes are relatively short and thick and show characteristic loops or cross-shaped configurations, he coined the name *diakinesis*, meaning 'moving apart', since he saw that the chromosomes were well-separated from one another at this stage, whereas in the first part of the prophase he thought, like many others at the time, that they were joined end-to-end. He illustrated the diakinetic stage from both plants and animals, using the published diagrams of others. Since it is now established that the various chromosomes are not joined end-to-end in early prophase I, the term diakinesis is really a misnomer. It is also out of keeping with von Winiwarter's terms (with the suffix *-tene*) for the earlier stages. In the diakinetic chromosomes the successive loops lie at right angles to one another, whereas at the diplotene stage they are all in one plane. Otherwise the chromosomes do not differ in any important respect from the diplotene ones, apart from being shorter and thicker (Fig. 6.2(*e*) and Pl. 3(*b*)), and sometimes with fewer loops (see §8.18).

Observation of diplotene or diakinetic nuclei in favourable material soon established that the structures within them are not merely double but quadruple (Pl. 3(*a*)). In other words, each chromosome, in addition to being associated with a counterpart, is itself divided into two chromatids. How the four strands which make up the diplotene and diakinetic chromosome pairs have originated has been a source of prolonged debate and will be discussed in Chapter 8.

§ 6.6 Later stages of meiosis

With the disappearance of the nucleoli and of the nuclear membrane, prophase I gives way to *metaphase I*. Each chromosome pair takes up a position on the equatorial plate of the spindle such that the centromeres of the two component chromosomes lie one on each side of the mid-plane (Fig. 6.2(*f*) and Pl. 3(*c*)). Thus, whereas in metaphase of mitosis the centromeres of the individual chromosomes lie on the equator of the spindle, at metaphase I of meiosis the centromeres of a chromosome-pair lie one in the tropic of Cancer and the other of Capricorn and both on the same line of longitude. At this stage, the chromosomes are exceptionally short and thick.

Anaphase I is said to begin when the two whole chromosomes forming each pair start to move apart towards opposite poles of the spindle. At this stage the two chromatids of which each chromosome is composed are readily seen (see Fig. 6.2(*g*)). Nuclear membranes develop round each group of

chromosomes at *telophase I* (Fig. 6.2(*h*)). After a brief *interphase*, when the chromosomes become difficult to observe, *prophase II* follows during which they become shorter and thicker again (Fig. 6.2(*i*)). *At metaphase II* the nuclear membranes have broken down again and two new spindles have formed (Fig. 6.2(*j*)). The second division of meiosis resembles mitosis in the centromeres of the chromosomes taking up a position on the equatorial plate, but differs in the two chromatids, of which each chromosome has been composed ever since prophase I, diverging somewhat from one another instead of being closely associated (Pl. 3(*e*)). At *anaphase II* the daughter-chromosomes separate and move towards opposite poles on each spindle with the centromeres as usual leading the way (Fig. 6.2(*k*)), and at *telophase II* nuclear membranes have formed round each of the four haploid nuclei which result from meiosis (Fig. 6.2(*l*)).

The essential features of meiosis which distinguish it from mitosis are, first, that whole chromosomes associate in pairs at prophase *I* and separate to opposite poles at anaphase *I*; and secondly, that there is no duplication of the chromosomes in the brief interphase between the two nuclear divisions, so that the daughter-chromosomes which separate at anaphase II were those first seen at prophase I. Thus, instead of the single nuclear division without chromosome division which Weismann (1887) had anticipated, when the details of meiosis were discovered, it was found that the process was less simple: as indicated above, there are two divisions of the nucleus associated with one of the chromosomes.

§ 6.7 Evidence that paternal and maternal chromosomes associate

Montgomery (1901), from studies of the chromosomes at meiosis in some 42 species of hemipterous insects, came to the conclusion that the chromosomes which associate in pairs at prophase I are of paternal and maternal origin respectively. His evidence for this important deduction was two-fold. First, whenever two chromosomes could be distinguished by their greater size, as for instance in *Protenor belfragei*, these two especially large ones were always found paired with one another at prophase I. Secondly, in *Euschistus variolarius* with 14 chromosomes visible at the somatic mitoses, 7 bivalent associations were always found at prophase I of meiosis. He argued that if chromosome association was paternal with paternal this would give 3 bivalent and 1 univalent association, and likewise with the chromosomes of maternal origin. The regular formation of pairs even when, as here, the haploid chromosome number is odd, led Montgomery to the conclusion that the bivalent associations at prophase I are always made up of one paternal and one corresponding maternal chromosome.

Sutton (1902) extended Montgomery's observations when he showed that in the grasshopper *Brachystola magna* all the chromosomes can be individually recognised. The somatic mitosis in the male showed 23 chromosomes which could be classified by size into 11 pairs and one odd one (the *X*-chromosome—see § 6.10). The largest chromosomes were 5 to 6 times as long as the smallest.

At prophase I of meiosis, apart from the *X*-chromosome, 11 double chromosomes were visible in each nucleus and they showed the same size differences as at mitosis. Moreover, though the absolute lengths of the chromosomes varied greatly at different stages of meiosis, their relative lengths were approximately constant. It was evident that each chromosome was distinct and maintained its morphological individuality throughout successive cell-divisions, and that the meiotic pairing was strictly between paternal and maternal homologues, as Montgomery had suggested.

§ 6.8 Evidence of qualitative differences between chromosomes

Boveri (1902) found evidence that the individual chromosomes in *Paracentrotus lividus* (a Sea Urchin) possess different qualities. This remarkable conclusion was based on studies of abnormal progeny resulting from fertilisation of eggs by two sperms. Boveri showed that the first mitosis in such *triploid* eggs, that is, with 3 sets of chromosomes, was abnormal, and gave rise to an irregular distribution of the chromosomes to the daughter-nuclei. He was able to show that for normal development, a particular combination of chromosomes was more important than a particular number. It will be recalled that he had shown earlier (see Chapter 5) that haploid individuals (from enucleated eggs) were normal although dwarf. He now established that they were normal because they had the normal combination of chromosomes. This important principle has subsequently been confirmed in many other organisms. Thus, Blakeslee (1922) showed that in *Datura stramonium* (Thorn Apple) triploid and *tetraploid* individuals, having respectively 3 and 4 complete sets of 12 chromosomes in their somatic cells, only differed rather slightly in appearance from the normal diploid, whereas individuals which he described as *trisomic*, having one of the chromosomes represented three times but the remaining 11 represented twice, were abnormal. The discovery of individuals with each of the 12 different chromosomes in turn present in triplicate revealed that each had a different morphology. Thus, three doses of one chromosome gave rise to a variety called Globe with broader leaves than normal, a less toothed leaf-margin, and a spherical instead of ovate capsule with shorter spines than normal (Fig. 6.3(*a*) and (*b*)).

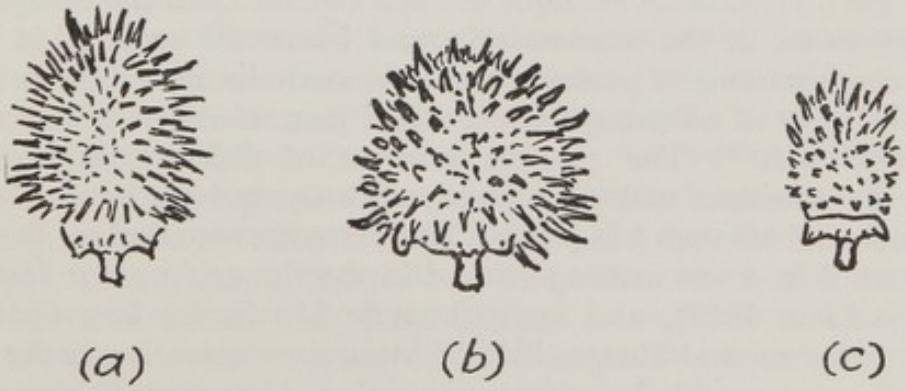


FIGURE 6.3 Fruits (×0·5) of normal and trisomic plants of *Datura stramonium* (from Blakeslee). (*a*) Normal. (*b*) Globe. (*c*) Cocklebur.

Individuals trisomic for a different chromosome gave a variety called Cocklebur with weak stems, narrow twisted leaves and a narrow short-spined capsule (Fig. 6.3(*c*)). Each trisomic variety differed from the normal in a whole range of characters affecting all parts of the plant.

Knowledge of the abnormality of trisomic individuals has recently been extended to man. From squash preparations of mitosis in cultures of lung tissue from 4 embryos of unknown sex after legal abortion, Tjio and Levan (1956) showed that the human somatic chromosome number was 46. Confirmation of this number was obtained by Ford and Hamerton (1956) from study of meiosis in fresh operative specimens of testis tissue from 3 individuals. Human chromosomes are readily classified by size into 7 classes, and within these classes the individual chromosomes can be recognised by the relative lengths of their arms (Pl. 1(*e*)). By international agreement, the largest of the 23 pairs is called no. 1, and the others have been allocated identification numbers in order of decreasing size. Lejeune, Gautier and Turpin (1959) discovered that mongolism is associated with the presence of an additional small chromosome. It appears that mongols have three editions of chromosome no. 21 in their somatic cells, instead of the normal two.

§ 6.9 The chromosome theory of Mendelian heredity

Sutton (1903), on the basis of his studies on *Brachystola magna* and with the support of Montgomery's and Boveri's findings, gave the first clear formulation of the chromosome theory of Mendelian heredity. He drew attention to the resemblance between the separation of homologous chromosomes at meiosis and Mendel's postulated separation of character-differences at gamete-formation. Moreover, Sutton pointed out that in all probability each homologous pair of chromosomes is orientated on the metaphase I spindle independently, so that any pair of chromosomes may lie with maternal or paternal chromosome indifferently towards either pole irrespective of the orientation of other pairs. He noted that this would lead to a large number of different possible combinations of maternal and paternal chromosomes in the gamete nuclei, if the chromosome number were at all large: indeed 2^n combinations, where n is the haploid chromosome number. He quoted as an example the Sea Urchin *Lytechinus variegatus* with 36 chromosomes in the somatic cells and hence 2^{18} or 262,144 possible gametic combinations of paternal and maternal chromosomes, so that the number of types of offspring from a single pair would be 2^{36} or approximately $6{\cdot}87 \times 10^{10}$! The possible number of different assortments of parental chromosomes will vary greatly with the species, because chromosome numbers show such a big range. The extremes recorded for the haploid number are 2 in a few arthropods and in the flowering plant *Haplopappus gracilis* (Jackson, 1957), and approximately 510 in the fern *Ophioglossum petiolatum* (Manton and Sledge, 1955). Sutton drew attention to the parallel between the presumed independent orientation of homologous chromosome-pairs at meiosis, and Mendel's observation of independent inheritance of different character-differences.

Confirmation of this random orientation of different chromosome-pairs on the first-division spindle was obtained by Carothers (1913). She examined 300 cells showing metaphase I or anaphase I of meiosis from males of *Brachystola magna* heterozygous for an inequality in the size of one of the chromosomes. She found that this size difference always segregated at the first division of meiosis. The single *X*-chromosome moves in its entirety to one pole at this division. In 146 of the cells, the smaller component of the unequal pair went to the same pole as the *X*-chromosome, and in the remaining 154 cells it was the larger component which segregated with the

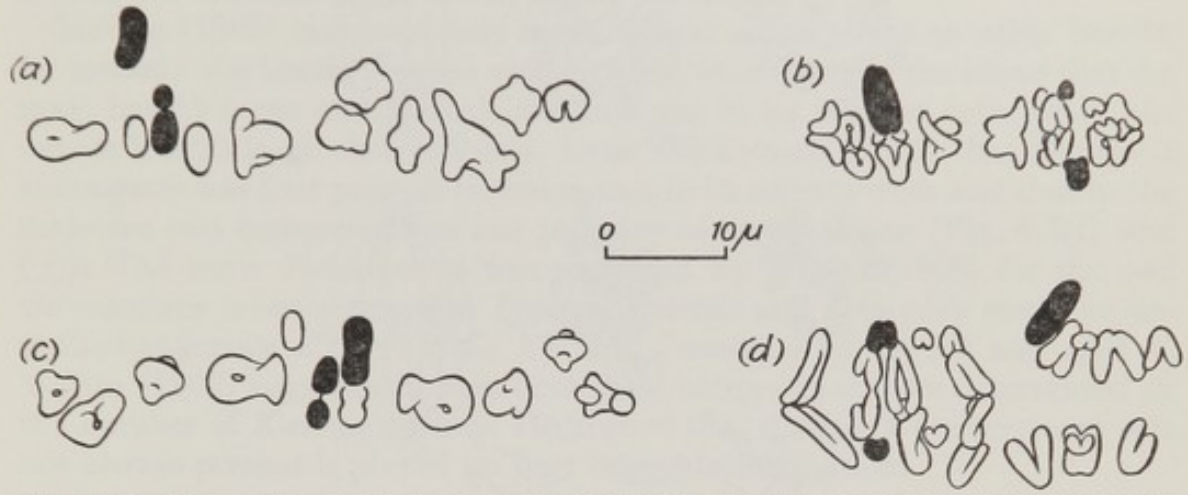


FIGURE 6.4 Metaphase I (*a* and *c*) and anaphase I (*b* and *d*) of meiosis in males of *Brachystola magna* heterozygous for an inequality in the size of one of the chromosomes (from Carothers). The unequal chromosome-pair and the *X*-chromosome are shown black. (*a* and *b*) Smaller component orientated to move to the same pole as the *X*-chromosome. (*c* and *d*) Larger component so orientated.

X-chromosome (see Fig. 6.4). Similar results were obtained with another grasshopper, *Arphia simplex*. Some exceptions to this random orientation of different chromosome-pairs on the meiotic first-division spindle are discussed in §18.3.

§ 6.10 Sex chromosomes

The first definite evidence for a connection between the chromosomes and a character-difference showing Mendelian inheritance came from work on sex-determination. Henking (1891) in studies on meiosis at sperm-formation in the hemipterous insect *Pyrrhocoris apterus* observed a deeply-staining chromatin-element, which passed to one pole on each anaphase II spindle, so that half the sperms received it and half did not (see Fig. 6.5(*a*) and (*b*)). He labelled it *X* as he was uncertain whether it was a chromosome or not. Later workers on insect cytology established its chromosomal nature, and after observing a similar unpaired chromosome in various grasshoppers, McClung (1902) suggested that it was concerned with sex-determination. This was established by Wilson (1905) who discovered that the single *X-chromosome* (as he called it, Wilson 1909) of the male *Protenor belfragei*

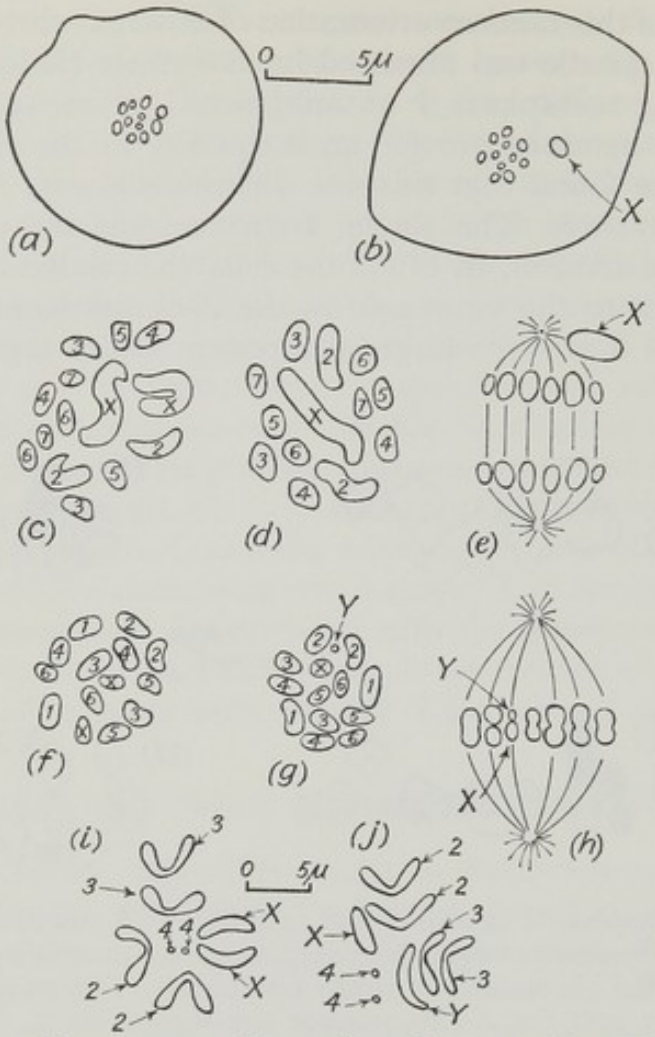


FIGURE 6.5 Sex chromosomes in various insects. X = X-chromosome. Y = Y-chromosome. Numbers refer to the autosomes in order of diminishing size

(*a* and *b*) *Pyrrhocoris apterus*. Polar views of the two groups of chromosomes on one of the spindles at anaphase II of meiosis in the male (from Henking, 1891). The single X-chromosome moves to one pole at anaphase II.

(*c–e*) *Protenor belfragei* (from Wilson).

(*c*) Female. Late prophase of mitosis in an oogonium. Two X-chromosomes present.

(*d*) Male. Late prophase of mitosis in a spermatogonium. A single X-chromosome present.

(*e*) Male. One spindle at anaphase II of meiosis. The single X-chromosome moves to one pole.

(*f–h*) *Lygaeus turcicus* (from Wilson).

(*f*) Female. Late prophase of mitosis in an oogonium. Two X-chromosomes present.

(*g*) Male. Late prophase of mitosis in a spermatogonium. An X- and a Y-chromosome present.

(*h*) Male. One spindle at metaphase II of meiosis. The X- and Y-chromosomes separate to opposite poles at anaphase II.

(*i* and *j*) *Drosophila melanogaster* (from Stevens).

(*i*) Female. Polar view of metaphase of mitosis in an oogonium. Two X-chromosomes present.

(*j*) Male. Polar view of metaphase of mitosis in a spermatogonium. An X- and a Y-chromosome present.

has two counterparts in the female, so that the somatic chromosome numbers of male and female are 13 and 14 respectively (Fig. 6.5(*c*)–(*e*)). He argued from these observations that the sperms which receive an *X*-chromosome will give rise to females and those that do not to males. Similar observations were made on several other hemipterous insects, while in a second group of these insects, exempified by *Lygaeus turcicus*, both sexes were found to have the same chromosome number ($2n = 14$ in this instance), but one chromosome in the male was much smaller than the corresponding one in the female (Fig. 6.5(*f*)–(*h*)). Again Wilson argued that there must be a causal connection between these chromosomes and sex.

Stevens (1905) independently made similar observations on other insects, for instance the beetle *Tenebrio molitor* (Meal worm) where she found that the male has 19 large and 1 small chromosome in its somatic cells, while the female has 20 large chromosomes. Later she showed (1908) that *Drosophila melanogaster* has four pairs of chromosomes in its somatic cells and that in the male the two components of one pair are of unequal size (Fig. 6.5(*i*) and (*j*)). The term *Y-chromosome* was proposed by Wilson (1909) for the odd chromosome when present, so *Lygaeus*, *Tenebrio* and *Drosophila* could be described as female *XX* and male *XY*, and *Protenor* as female *XX* and male *X*. Wilson (1909) suggested that in both these categories sex was determined by the number of *X*-chromosomes. He argued that since a *Y*-chromosome was not always present it played no part in sex-determination.

§ 6.11 The chromosome theory of sex-linkage

On the basis of Wilson's theory of sex-determination, Morgan (1911*a*) postulated that the gene for white eyes in *Drosophila melanogaster* is carried by the *X*-chromosome. This hypothesis to explain the sex-linkage which he had found (see Chapter 3) represented an important advance over his earlier theory (based on that of Punnett and Bateson for *Abraxas*), since it was no longer necessary to assume that all male Drosophilas were heterozygous for white eye-colour, for, with only one *X*-chromosome in the male, heterozygosity was impossible for sex-linked characters. Moreover the criss-cross inheritance of the X-chromosomes was exactly paralleled by that of white eye-colour (see Figs 6.6 and 6.7).

The sex-linkage of the pale form of *Abraxas grossulariata* was similarly explained when it was established that in Lepidoptera it is the female which has either an *X*- and a *Y*-chromosome, or an unpaired *X*-chromosome, while the male has 2 *X*-chromosomes, that is, the converse of the situation in *Drosophila*.

Red-green colour blindness in man was found to show criss-cross inheritance similar to white eye-colour in *Drosophila*. That is to say, colour-blindness is usually manifest only in males, whose daughters act as carriers of it to half their sons. The female *XX*, male *XY* sex-determining mechanism in man was confirmed subsequently (cf. Pl. 1(*e*)). By contrast, the dominant character barred feather-colouring of the Barred Plymouth Rock and its recessive allele the black feather-colouring of the Black Langshan variety of

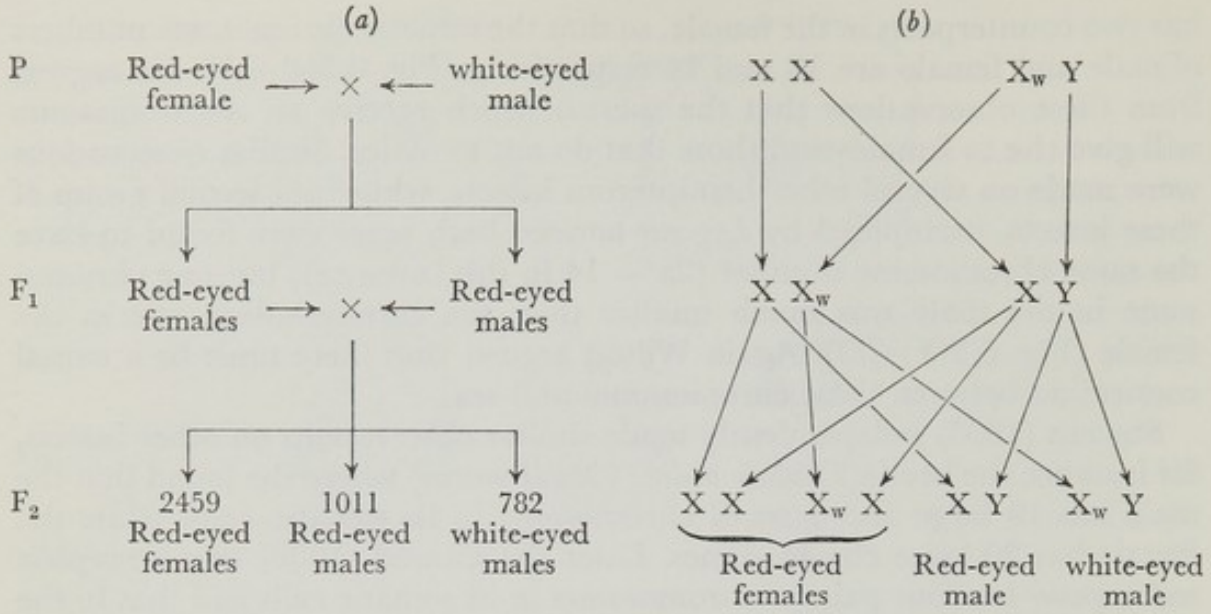


FIGURE 6.6 (*a*) Morgan's data (1910) for the inheritance of white eye-colour in the normally red-eyed fly *Drosophila melanogaster*.
(*b*) The hypothesis which he proposed (1911*a*) to account for these results, namely, that the gene for white eyes is carried by the *X*-chromosome. Explanation of symbols:
X = *X*-chromosome with dominant normal allelomorph of white-eye gene, and hence causing red eyes.
X_w = *X*-chromosome with recessive gene for white eyes.
Y = *Y*-chromosome (plays no part in inheritance).
Two *X*-chromosomes give a female, one *X*-chromosome a male. The shortage of males in F_2, particularly those with white eyes, compared with expectation, can probably be accounted for by lowered viabilities.

Gallus domesticus were found to show sex-linkage of the *Abraxas* type, and the cytology confirmed that birds, like Lepidoptera, have the female *XY*, male *XX* sex-determining mechanism.

Baur (1912) obtained the first example of sex-linkage in plants. He discovered a form of *Silene alba* (White Campion) with narrow grass-like

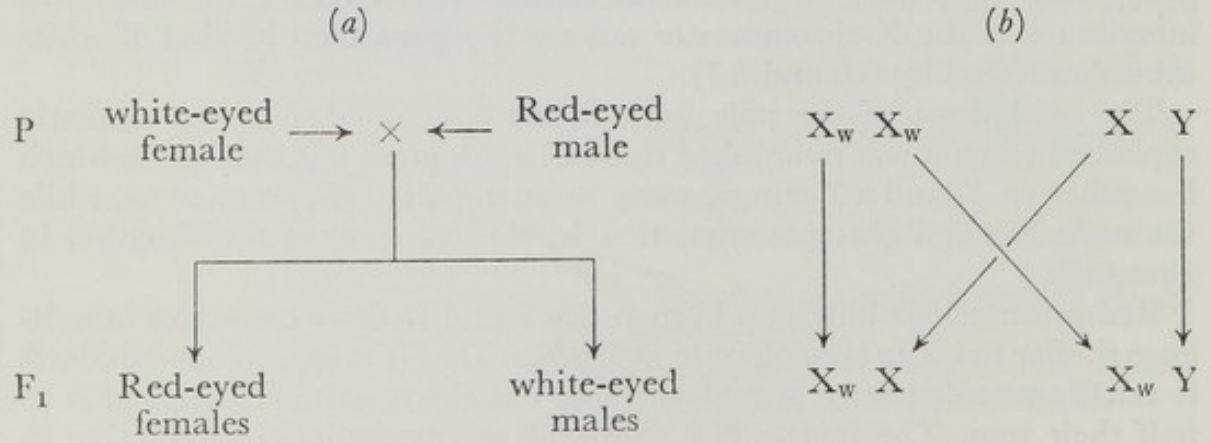


FIGURE 6.7 (*a*) Further data from Morgan (1910) concerning the inheritance of white eye-colour in *Drosophila melanogaster*, and
(*b*) his chromosome hypothesis (1911*a*) to account for them. Symbols as in Fig. 6.6.

leaves. It was found to be a staminate plant. Pollen from it was used to fertilize a female plant with normal broad leaves. All the progeny were normal, and when intercrossed they gave 167 plants with broad leaves and 60 with narrow leaves, in agreement with the Mendelian expectation of a 3:1 ratio (expected frequencies 170·25 and 56·75, deviations 3·25, $\chi^2 = 0{\cdot}24$, $n = 1$, $P = 0{\cdot}9$–$0{\cdot}5$). When 52 of the narrow-leaved plants were grown to maturity all were found to be male, while 23 of the broad-leaved ones comprised 15 female and 8 male, in good agreement with the 2:1 ratio of the sexes expected in F_2 individuals with the dominant phenotype, if *Silene alba* is like *Drosophila* in its sex-linkage (compare Fig. 6.6), that is, female *XX*, male *XY*, with the gene for narrow leaves carried by the *X*-chromosome. Subsequently, cytological studies confirmed that *Silene* has this chromosome formula.

§ 6.12 *XXY Drosophila*

The chromosome theory of Mendelian heredity may be said to have become established with the publication of the results of combined cytological and genetical studies by Bridges (1914, 1916) with an abnormal strain of *Drosophila melanogaster* having two *X*-chromosomes and a *Y* chromosome.

Morgan (1910) had reported that a white-eyed female *Drosophila* crossed with a normal red-eyed male always gave equal numbers of the converse types in F_1, that is, red-eyed females and white-eyed males. On the hypothesis that the gene for white eyes is carried by the *X*-chromosome these results were readily explained (Morgan 1911*a*) by the criss-cross inheritance of the *X*-chromosomes, as indicated in Fig. 6.7. However, Morgan and Bridges discovered a strain of white-eyed females which gave some unexpected progeny from such a cross. These unexpected individuals comprised about 4·3% of the progeny, and consisted of approximately equal numbers of the two parental forms (white-eyed females and red-eyed males). The occurrence of these *matriclinous* daughters (that is, resembling their mother) and *patriclinous* sons (resembling their father) appeared to contradict the chromosome hypothesis.

Bridges studied the progeny in the succeeding generation, with the following results. The matriclinous daughters (white eyed; class (i) in Table 6.2) behaved like their mother and again gave about 4.3% of parental forms when crossed with normal red-eyed males. The patriclinous sons (red-eyed; class (ii) in Table 6.2) when crossed with unrelated females gave no abnormal progeny in the next or succeeding generations. Half of the ordinary patriclinous daughters (red-eyed; class (iii)) gave normal progeny indefinitely, and the other half (class (iv)) inherited the property of giving about 4·3% of unexpected progeny. Half of the ordinary matriclinous sons (white eyed; class (v)) gave normal progeny indefinitely and the other half (class (vi)) transmitted the property of producing exceptional progeny to some of their daughters, although not themselves having this property. These results are summarised in Table 6.2(*a*).

To account for these results, Bridges postulated that the original white-eyed female was abnormal in possessing a *Y*-chromosome in addition to two

TABLE 6.2 (*a*) Bridges' results from crossing an abnormal white-eyed female *Drosophila melanogaster* with a normal Red-eyed male, (*b*) his total data (1916) from this and similar crosses using various sex-linked mutant characters, and (*c*) his hypothesis to explain these results. Symbols as in Fig. 6.6.

(*a*)

Parents	Red-eyed (normal) male	
White-eyed female from abnormal stock		2·1% of white-eyed females (i), all with some exceptional progeny.
	2·2% of Red-eyed males (ii), all with normal progeny.	
	49·0% of Red-eyed females, half (iii) with normal progeny, and half (iv) with some exceptional progeny.	46·7% of white-eyed males, half (v) with normal progeny, and half (vi) with some exceptional progeny.

(*b*)

Parents	Normal males	
Females from abnormal stock, with various sex-linked mutant characters		1169 females of mutant appearance (i).
	1235 males of normal appearance (ii).	
	27,679 females of normal appearance (iii and iv).	26,391 males of mutant appearance (v and vi).

(*c*)

Parents			*XY* (Red-eyed male)	
			Sperm	
			X	*Y*
X_wX_wY (white-eyed female from abnormal stock)	Eggs 8·2%	X_wX_w	XX_wX_w Dies	(i) X_wX_wY
		Y	(ii) XY	YY Dies
	Eggs 91·8%	X_w	(iii) XX_w	(v) X_wY
		X_wY	(iv) XX_wY	(vi) X_wYY

X-chromosomes. Furthermore he postulated that at the first division of meiosis in the maturation of the eggs there was variability of behaviour such that in about 92% of meioses one *X*-chromosome and the *Y*-chromosome went to one pole and the remaining *X*-chromosome to the other pole, while in the other 8% the two *X*-chromosomes separated from the *Y*. This hypothesis will account perfectly for all the observations, as indicated in Table 6.2(*c*). Individuals with 0 or 3 *X*-chromosomes were thought not to survive.

Confirmation of this *XXY* hypothesis was obtained from cytological studies: Bridges found that the white-eyed females and half the red-eyed ones had two *X*-chromosomes and a *Y*-chromosome, exactly as the theory predicts. It is presumed that the original *XXY* female arose through fertilisation by a *Y* sperm of an abnormal egg containing two *X*-chromosomes. This egg might have been the consequence of a meiotic abnormality whereby both *X*-chromosomes passed to the same pole of the spindle. Bridges (1916) reported several instances of this failure of the *X*-chromosomes to separate at meiosis even in the absence of a *Y*-chromosome. Altogether he bred 56,474 F_1 progeny from *XXY* females carrying various sex-linked characters crossed to normal males, with the results shown in Table 6.2(*b*). The percentages in Table 6.2(*a*) are based on these results.

§ 6.13 Sex determination

In addition to establishing the truth of the chromosome theory of Mendelian inheritance, Bridges' experiments with *XXY* flies also confirmed Wilson's theory of sex-determination, for it was evident that an *XXY* fly was female and an *XYY* fly male, as expected if sex is determined by the number of *X*-chromosomes irrespective of the number of *Y*-chromosomes. Bridges (1922) later showed that sex is determined by the proportion of *X*-chromosomes to other chromosomes (*autosomes*). If *A* stands for a haploid set of autosomes and *X* for an *X*-chromosome, $\frac{X}{A}$ is $\frac{1}{2}$ in the normal male and $\frac{2}{2}$ in the normal female. With three *X*-chromosomes $\left(\frac{X}{A} = \frac{3}{2}\right)$ abnormal sterile females of low viability and delayed development were obtained. Bridges also obtained triploid flies with three sets of autosomes and found that $\frac{X}{A} = \frac{1}{3}$ gave an abnormal sterile male of slow development, $\frac{X}{A} = \frac{2}{3}$ gave a sterile fly of appearance intermediate between the two sexes, while $\frac{X}{A} = \frac{3}{3}$ gave a female of almost normal appearance, although sterile. Thus his results were consistent with the view that sex was determined by the proportion of *X*-chromosomes and autosomes, which were presumed to carry genes for female and male characters, respectively. Later work has confirmed that, irrespective of the number of *Y*-chromosomes, $\frac{X}{A}$ of $\frac{1}{2}$ or less gives males, 1 or more gives females, and between $\frac{1}{2}$ and 1 gives intermediates.

On the other hand, in *Silene alba*, *Homo sapiens* and *Mus musculus* a different mechanism appears to operate. Westergaard (1958) in reviewing his own and others' work with *Silene* showed that the presence of the *Y*-chromosome gives a male and its absence a female, irrespective of the number of *X*-chromosomes and sets of autosomes. The only exceptions are tetraploid plants with 4 *X*-chromosomes but only 1 *Y*-chromosome, which are usually hermaphrodite. It appears that the male-determining genes reside on the *Y*-chromosome and the female-determining on the *X*-chromosome, and that a ratio of $4X:1Y$ is required even to produce an hermaphrodite. Work with fragmented *Y*-chromosomes suggests that this chromosome contains genes which lead to the development of male characters and others which suppress the development of female characters.

In *Homo sapiens* and *Mus musculus* normal females have two *X*-chromosomes and normal males an *X*- and a *Y*-chromosome. Compared with the other human chromosomes, the *X*-chromosome is in the third class for size, while the *Y*- is in the seventh or smallest class (Pl. 1(*e*)). In both *Homo* and *Mus* an abnormal condition occurs occasionally where there is a single *X*-chromosome and no *Y*-chromosome in the somatic nuclei. Such individuals are female, and fertile in *Mus* (Welshons and Russell, 1959), but usually sterile and with impaired development in *Homo* (Turner's syndrome). Individuals with two *X*-chromosomes and a *Y*-chromosome are sterile males, both in *Mus* (Russell and Chu, 1961) and *Homo* (Jacobs and Strong, 1959). In man, such individuals have certain slight female characteristics (Klinefelter's syndrome). It thus appears that, as in *Silene*, sex in these mammals is determined by the proportion of *X*- and *Y*-chromosomes. An extra *Y*-chromosome in the human male seems often to cause the individual to be unusually tall, aggressive and mentally subnormal (Jacobs *et al.*, 1965) and to show from an early age criminal behaviour against property rather than persons (Price and Whatmore, 1967).

§ 6.14 Conclusion

Although Bridges' work on *XXY Drosophila* may be said finally to have established the truth of the chromosome theory of Mendelian heredity, nevertheless the theory still met opposition. This was primarily because it was found necessary to postulate that the homologous chromosomes regularly exchanged segments at meiosis, a notion which some biologists thought conflicted with the concept of the individuality and permanence of the chromosomes. This will be discussed in the next chapter.

7. *The theory of chromosomal crossing-over*

§ 7.1 De Vries' theory of factor exchange

In his formulation of the chromosome theory of Mendelian heredity, Sutton (1903) pointed out that the number of distinct characters in an organism must exceed the number of chromosomes. He argued from this that the basis of an allele was only a part of a chromosome, and that if the chromosomes permanently retain their individuality all the factors represented by one chromosome must be inherited together. However, within a short time, four apparently independently-inherited character-differences were known in *Pisum sativum* in addition to the seven studied by Mendel, and yet the haploid chromosome number was known to be only seven. Similarly, the number of apparently freely-inherited character-differences in *Antirrhinum majus* was found to exceed the haploid chromosome number of 8. These findings appeared to be a fatal objection to the chromosome theory. However, De Vries (1903) had proposed a theory which offered a solution to this dilemma.

De Vries accepted Sutton's view that the chromosomes formed the material basis of Mendelian heredity, and that many factors must co-exist in each chromosome, but to account for the apparently independent inheritance of each character-difference he supposed that an exchange of material took place between the maternal and paternal homologous chromosomes while they were closely associated in pairs during prophase I of meiosis. He argued that if two like factors lay opposite each other, a simple exchange of factors could occur. He assumed that in any given instance it was a matter of chance whether particular factors were exchanged or not. He argued further that a factor could be exchanged only for a like one, that is, for one which represented the corresponding hereditary character, otherwise each chromosome would not retain the entire series of factors. He regarded it as essential that the progeny should receive from their parents the sum total of these factors, as representing all the characters of the species. He pointed out that after these hypothetical exchanges had occurred, each chromosome would contain some paternal and some maternal units. Furthermore, since the two chromosomes of a pair pass into different gamete-nuclei, this redistribution of factors between the homologous chromosome-pairs would lead to the formation of gametes with all possible combinations of the parental character-differences, just as Mendelian theory required.

§ 7.2 Morgan's evidence for crossing-over

De Vries' theory of exchanges between homologous chromosomes gained little support at the time. In the first place there was no evidence that such exchanges occurred, and secondly the idea of exchanges was thought by many to be contrary to the concept of the permanence and individuality of the chromosomes. For the same reasons, when Bateson, Saunders and Punnett (1905) discovered partial linkage between two different characters in *Lathyrus odoratus* (see Chapter 3), they did not favour the chromosome theory as an explanation. But when Morgan (1911*a*), working with *Drosophila*

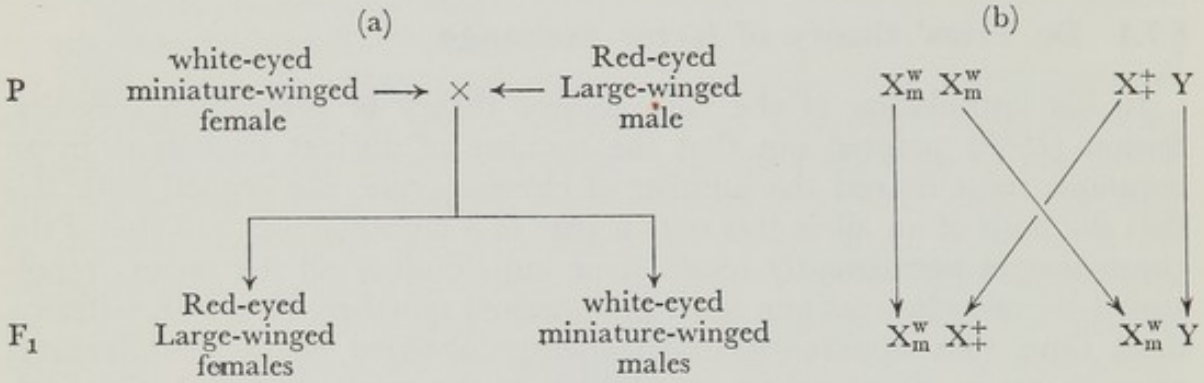


FIGURE 7.1 Morgan's results from crossing a white-eyed miniature-winged female with a normal male *Drosophila melanogaster*.
(*a*) F_1 results.
(*b*) Hypothesis to explain (*a*).
X^w_m = *X*-chromosome with recessive genes for white eyes (*w*) and miniature wings (*m*).
X^+_+ = *X*-chromosome with dominant normal allelomorphs *w* and *m*.
Y = *Y*-chromosome.

melanogaster, turned up a similar example of partial linkage, he had no alternative but to postulate a De Vriesian exchange of material between homologous chromosomes, because the characters concerned, white eye-colour and miniature wings, both showed sex-linkage. If the genes for each of these characters were carried by the *X*-chromosome, as the sex-linkage data suggested, then the only way to explain the occurrence of new combinations of them was to postulate exchanges between the two *X*-chromosomes (in the female insect, since the male has only one *X*-chromosome). Thus, when he crossed a white-eyed miniature-winged female with a normal (Red-eyed Large-winged) male, the F_1 progeny were similar except that the sexes were reversed, that is, the females were all normal and the males all white-eyed and miniature-winged (see Fig. 7.1). This result is what is expected (see Chapter 6) if the genes for white eyes and miniature wings are both carried by the *X*-chromosomes (see Fig. 6.7). The F_1 flies were allowed to interbreed, and 2441 progeny (F_2) obtained (Morgan 1911*b*). The numbers of each kind are shown in Table 7.1(*a*). It will be noticed that flies with the grandparental character-combinations (white-eyed miniature-winged and Red-eyed Large-winged) are the most frequent in both sexes (classes (i) to (iv)).

The surprising discovery, however, was the occurrence of appreciable numbers of flies with the other two combinations of the eye and wing characters (classes (v) to (viii)). Altogether there were just 900 of these out of the 2441 or 36·9%. Morgan's hypothesis to explain these results is shown in

TABLE 7.1 Morgan's results from crossing a white-eyed miniature-winged female with a normal male *Drosophila melanogaster*.
(*a*) F_2 results.
(*b*) Hypothesis to explain (*a*).
Symbols as in Fig. 7.1.

(*a*)

Parents(F_1)	White-eyed Miniature-winged Males					
	Eyes	Wings	Females	Males	Totals	
Red-eyed Large-winged females	white	miniature	(i) 359	(ii) 391	750	1541
	Red	Large	(iii) 439	(iv) 352	791	
	white	Large	(v) 218	(vi) 237	455	900
	Red	miniature	(vii) 235	(viii) 210	445	
	Totals		1251	1190		2441

(*b*)

Parents (F_1) and their Gametes				$X_m^w Y$	
				Sperm	
				X_m^w	Y
$X_m^w X_+^+$	Eggs	63·1%	X_m^w	(i) $X_m^w X_m^w$	(ii) $X_m^w Y$
			X_+^+	(iii) $X_+^+ X_m^w$	(iv) $X_+^+ Y$
		36.9%	X_+^w	(v) $X_+^w X_m^w$	(vi) $X_+^w Y$
			X_m^+	(vii) $X_m^+ X_m^w$	(vii) $X_m^+ Y$

Table 7.1(*b*). He assumed that in 36·9% of the eggs an exchange of factors had occurred, just as De Vries had anticipated, between the two *X*-chromosomes.

Another Mendelian character in *Drosophila* which Morgan found to show sex-linkage was yellow body-colour. When he crossed a white-eyed yellow-bodied female with a normal (Red-eyed Grey-bodied) male, the F_1 progeny comprised normal females and white-eyed yellow-bodied males, as expected with sex-linkage (Fig. 7.2). When the F_1 flies were intercrossed, 2205 F_2 flies were obtained. The numbers of each kind are shown in Table 7.2. Again it is flies with the grandparental character-combinations (white-eyed yellow-bodied and Red-eyed Grey-bodied) which were most frequent, but

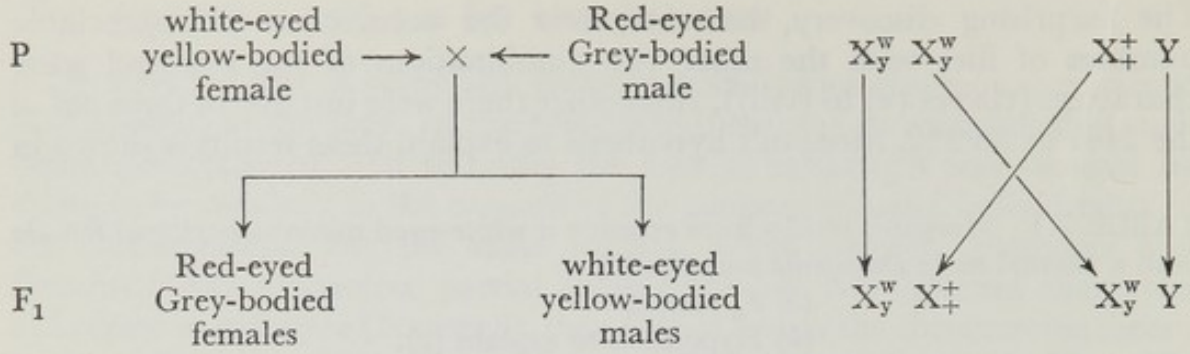


FIGURE 7.2 Morgan's results from crossing a white-eyed yellow-bodied female with a normal male *Drosophila melanogaster*.
(a) F_1 results.
(b) Hypothesis to explain (*a*).
X_y^w = *X*-chromosome with recessive genes for white eyes (*w*) and yellow body (*y*).
X_+^+ = *X*-chromosome with dominant normal allelomorphs of *w* and *y*.
Y = *Y*-chromosome.

TABLE 7.2 Morgan's results from crossing a white-eyed yellow-bodied female with a normal male *Drosophila melanogaster*.
(*a*) F_2 results.
(*b*) Hypothesis to explain (*a*).
Symbols as in Fig. 7.2.

(*a*)

Parents (F_1)	White-eyed Yellow-bodied Males					
	Eye-colour	Body-colour	Females	Males	Totals	
Red-eyed Grey-bodied females	white	yellow	(i) 543	(ii) 474	1017	2176
	Red	Grey	(iii) 647	(iv) 512	1159	
	white	Grey	(v) 6	(vi) 11	17	29
	Red	yellow	(vii) 7	(viii) 5	12	
		Totals	1203	1002		2205

(*b*)

Parents (F_1) and their Gametes			$X_y^w Y$	
			Sperm	
			X_y^w	Y
$X_y^w X_+^+$	Eggs	98·7% X_y^w	(i) $X_y^w X_y^w$	(ii) $X_y^w Y$
		98·7% X_+^+	(iii) $X_+^+ X_y^w$	(iv) $X_+^+ Y$
		1·3% X_+^w	(v) $X_+^w X_y^w$	(vi) $X_+^w Y$
		1·3% X_y^+	(vii) $X_y^+ X_y^w$	(viii) $X_y^+ Y$

this time the other two combinations of these character-differences appeared only infrequently: 29 out of 2205, or 1·3%. Morgan concluded that the factors responsible for these character-differences tended to remain together much more often than those in the previous experiment. This principle of partial linkage he called the *third law of heredity* (Morgan 1919), the first two laws being those of Mendel (see Chapter 3).

Morgan (1911*b*) reported results for over 9000 F_2 flies from crosses involving these characters (white eyes, miniature wings and yellow body) in different combinations. It was evident that the grandparental combinations were always the most frequent. Thus, when a homozygous Red-eyed yellow-bodied female was crossed with a white-eyed Grey-bodied male, the male F_2 progeny comprised 349 with Red eyes and yellow bodies and 374 with white eyes and Grey bodies (the grandparental combinations), while there were just two of each of the other combinations. It was the latter which were so frequent in the F_2 progeny from the cross described in the last paragraph, where these were the grandparental combinations. Morgan concluded that during segregation of character-differences at meiosis certain genes tend to remain together, not because of any attraction between them but because they lie near together in the same chromosomes. Furthermore, he argued that characters remain associated together to a greater extent the nearer the corresponding genes are together in the chromosome. The following year Morgan and Cattell (1912) introduced the term *crossing-over* for the process of interchange by which new combinations of linked factors arise.

§ 7.3 Linkage maps

Sturtevant (1913) took Morgan's argument a stage further. He pointed out that the proportion of cross-overs, that is, progeny derived from gametes in which an exchange had occurred, could be used as an index of the distance between any two genes. Morgan had already shown that this proportion was approximately constant for any particular genes, irrespective of their initial combinations. Thus, he had found that white *versus* Red eyes showed about 1% of cross-overs with yellow *versus* Grey body, whether initially the factors were in *coupling* (dominants in one parent, recessives in the other) or in *repulsion* (one dominant and one recessive in each parent). Sturtevant suggested that the unit of distance between two factor-pairs should be taken to be of such length that, on the average, one cross-over will occur in it out of every 100 gametes formed. In other words, he proposed that the percentage of cross-overs should be used as the index of distance. This concept of distance led him to an important discovery, namely, that the distances between a series of linked factors were additive, implying that the factors could be represented by a linear map. Thus, Morgan's F_2 data (1911*b*) from a cross between a yellow-bodied white-eyed miniature-winged female and a normal male gave 1·3% of cross-overs for the body-colour and eye-colour characters, 32·6% for eye-colour and wing-size, and 33·8% for body-colour and wing-size (see Table 7.3). The latter percentage is very

TABLE 7.3 Data of Morgan (1911b) for the F_2 progeny of a cross between a yellow-bodied white-eyed miniature-winged female and a normal male *Drosophila*. The shortage of yellow-bodied flies compared with the normal Grey in each complementary pair is probably due to a lower viability.

Characters			Number	Cross-overs		
Body colour	Eye colour	Wing size		Body colour and eye colour	Eye colour and wing size	Body colour and wing size
Grey	Red	Large	758	—	—	—
yellow	white	miniature	700	—	—	—
Grey	Red	miniature	401	—	401	401
yellow	white	Large	317	—	317	317
Grey	white	miniature	16	16	—	16
yellow	Red	Large	12	12	—	12
Grey	white	Large	1	1	1	—
yellow	Red	miniature	0	0	0	—
		Totals	2205	29	719	746
		Percentage	100	1·3	32·6	33·8

close to the sum of the other two and implies that the eye-colour factors lie between the others. The linkage map, using the percentages as distances, is shown in Fig. 7.3(*a*). Sturtevant pointed out that there was no means of knowing whether or not the distances as drawn represent the actual spatial distances, because some parts of the chromosome might be more liable to exchange than others. However, the ability to represent the factors by a linear map provided a new argument in support of the chromosome theory, since the chromosome was itself of thread-like proportions. The principle of the linear order of genes was called by Morgan (1919) the *fourth law of heredity*.

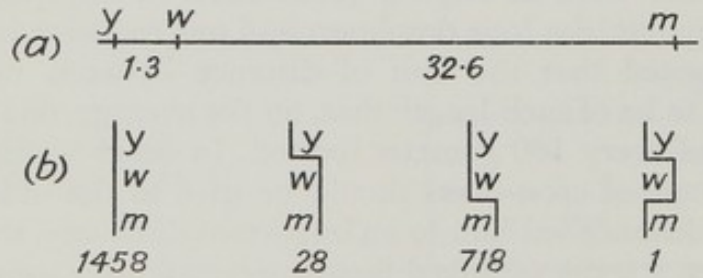


FIGURE 7.3 (*a*) Linkage map of the factors for yellow body (*y*), white eyes (*w*), and miniature wings (*m*) in *Drosophila melanogaster* derived from Morgan's data (1911*b*). (*b*) Summary of the data in Table 7.3 on the basis of the linkage map in (*a*). The lines indicate the position of the exchanges.

§ 7.4 Double crossing-over

Sturtevant found that the additive property of cross-over frequencies did not hold if all three frequencies were large. He attributed this discrepancy to double cross-overs. The data in Table 7.3 illustrate the effect of such double exchanges. There were 29 exchanges between the factors for body-colour and eye-colour and 719 between those for eye-colour and wing-size, making a total of 748, but between body-colour and wing-size there appeared to be only 746. The discrepancy is due to the single Grey-bodied white-eyed Large-winged fly. In the production of the egg from which this fly developed two exchanges must have occurred between the X-chromosomes, with the result that the body-colour and wing-size factors ('Grey' and 'Large', respectively) appeared together as in the grandparent, although the intervening factor (white eyes) was derived from the other grandparent (see Fig. 7.3(*b*)). It is evident that large cross-over frequencies give an inaccurate map, since, unless intervening factors are available, there will be no means of detecting double cross-overs. Various empirical formulae have been devised to allow for the effects of double crossing-over by 'correcting' recombination frequencies to give 'map units'.

In the light of these findings by Morgan and Sturtevant, earlier observations by others took on a new significance. Thus, characters due to factors placed far apart in a chromosome would evidently assort almost at random in the gametes, owing to the frequent occurrence of crossing-over between them. Here was evidently the explanation of how the number of apparently freely-inherited character-differences could exceed the haploid chromosome number.

§ 7.5 Test-crosses

The characters in *Lathyrus odoratus* found by Bateson, Saunders and Punnett (1905) to show 12% of cross-over gametes (Purple *versus* red flower colour and Long *versus* round pollen-shape) (see Chapter 3) could now be interpreted as due to factors at a distance apart of 12 units (or slightly more allowing for double cross-overs) on one of the chromosomes. Although *L. odoratus* was thus the first plant (or indeed organism of any kind) for which linkage map data became available, it is not a particularly favourable organism for linkage studies. This is because self-fertilisation (F_2) data are not easily interpreted (see Table 3.2). In studying linkage it is preferable to cross the heterozygous F_1 individuals with the homozygous recessive, since the relative frequencies of the different kinds of gametes from the F_1 heterozygote are then reflected directly in the frequencies of different kinds of progeny. Such a cross is called a *test-cross*. In *L. odoratus*, cross-fertilisation is tedious and gives few seeds per pollination. An ideal plant for linkage studies was found in *Zea mays*. In this grass the male and female flowers are widely separated at the apex and near the base of the plant, respectively, with the result that controlled cross-pollinations are readily made and moreover, several hundred seeds are normally obtained from a single pollination.

The first example of linkage in *Zea* was found by Collins (1912) between two endosperm characters: the colour of the aleurone layer and the horny (= starchy) or waxy texture of the endosperm tissue. An aleurone layer with a purplish pigment was found to be dominant to a colourless aleurone, and

TABLE 7.4 Data of Bregger (1918) for coupling and repulsion test-crosses with Coloured *versus* colourless aleurone (C/c) and Starchy *versus* waxy endosperm (*Wx*/*wx*) in *Zea mays*.

(*a*) Primary data.

(*b*) Totals for contingency χ^2 test (see text).

(*a*)

Testcross	Coloured Starchy	Colourless Waxy	Coloured Waxy	Colourless Starchy	Total
Coupling	147	133	65	58	403
Repulsion	46	32	103	111	292

(*b*)

Testcross	Parental	Recombinant	Total
Coupling	280	123	403
Repulsion	214	78	292
Total	494	201	695

the normal horny (starchy) endosperm giving a translucent grain was dominant to the opaque waxy endosperm, which had been found to occur in a maize variety from China. Several genes affect the aleurone pigmentation, and the first straightforward test-cross data for the waxy character and the colour factor showing linkage to it were obtained by Bregger (1918). He crossed a pure-breeding strain having Coloured aleurone and Starchy endosperm (*C C Wx Wx*) with one having colourless aleurone and waxy endosperm (*c c wx wx*). The doubly heterozygous F_1 plant so obtained had the factors in coupling $\left(\frac{C\ Wx}{c\ wx}\right)$. It was test-crossed with the strain having colourless aleurone and waxy endosperm (*c c wx wx*), and gave an ear with 403 grains, details of which are given in Table 7.4(*a*). There were 280 grains with the grandparental character combinations but only 123 with the other two combinations. The cross-over frequency is therefore 30·5%.

Bregger also crossed a pure-breeding strain having Coloured aleurone and waxy endosperm (*C C wx wx*) with one having colourless aleurone and Starchy endosperm (*c c Wx Wx*), and test-crossed the doubly heterozygous

F_1 progeny, which had the factors in repulsion $\left(\frac{C\ wx}{c\ Wx}\right)$, with the homozygous recessive strain (*c c wx wx*). From this cross he obtained an ear with 292 grains, the details of which are also given in Table 7.4(*a*). Again the grand-parental forms were recovered with higher frequency than the others, and the cross-over frequency is 26·7%. The results of the two test-crosses are set out in Table 7.4(*b*). Application of the contingency χ^2 test (see Chapter 4) gives

$$\chi^2 = \frac{695[(280 \times 78) - (214 \times 123)]^2}{403 \times 292 \times 494 \times 201} = 1{\cdot}2, \qquad n = 1,$$

and from Table 4.2 (p. 27), $P = 0{\cdot}5$–$0{\cdot}1$. Hence, the two estimates of the cross-over frequency do not differ significantly. It might be thought that the best estimate of the recombination frequency would be obtained simply from the total data, that is, $\frac{201}{695}$ or 28·92%. However, Muller (1916) pointed out that when coupling and repulsion linkage data are available, the effects of differential viabilities can be minimised by calculating the geometric mean of the ratios of recombinant to parental progeny from each cross. In the present instance, these ratios are $\frac{123}{280}$ and $\frac{78}{214}$, and their geometric mean is

$$\sqrt{\frac{123}{280} \times \frac{78}{214}} = 0{\cdot}4001.$$

If there are 40·01 recombinants to every 100 parental genotypes, the recombination frequency is $\frac{40{\cdot}01}{140{\cdot}01} = 28{\cdot}58\%$.

A third character of the endosperm of *Zea mays*, namely, a shrunken condition such as gives a concave surface to the apex or side of the mature grain, instead of the normal convex surface, was found to be determined by a recessive gene, *sh*, which showed linkage to *C/c* and *Wx/wx*. Stadler (1926) made extensive studies using these three linked character-differences. In one of these experiments the triple heterozygote (with all three factors in coupling) was crossed as female parent with a homozygous recessive plant as male parent, thereby forming a 'three-point test cross'. The 45,832 grains on 63 progeny plants were classified in respect of the three pairs of characters and the results are given in Fig. 7.4(*a*). From the data, the frequency (p) of recombination between *C/c* and *Sh/sh* is $\frac{1033 + 32}{45832} = 2{\cdot}32\%$. Similarly, the frequency (q) of recombination between *Sh/sh* and *Wx/wx* is $\frac{9109 + 32}{45832} = 19{\cdot}94\%$, and the frequency ($r$) of recombination between *C/c* and *Wx/wx* is $\frac{1033 + 9109}{45832} = 22{\cdot}13\%$. On the basis of these frequencies, it is evident that the locus of the gene for shrunken endosperm lies between the other two. The linkage map is shown in Fig. 7.4(*b*). As in the *Drosophila*

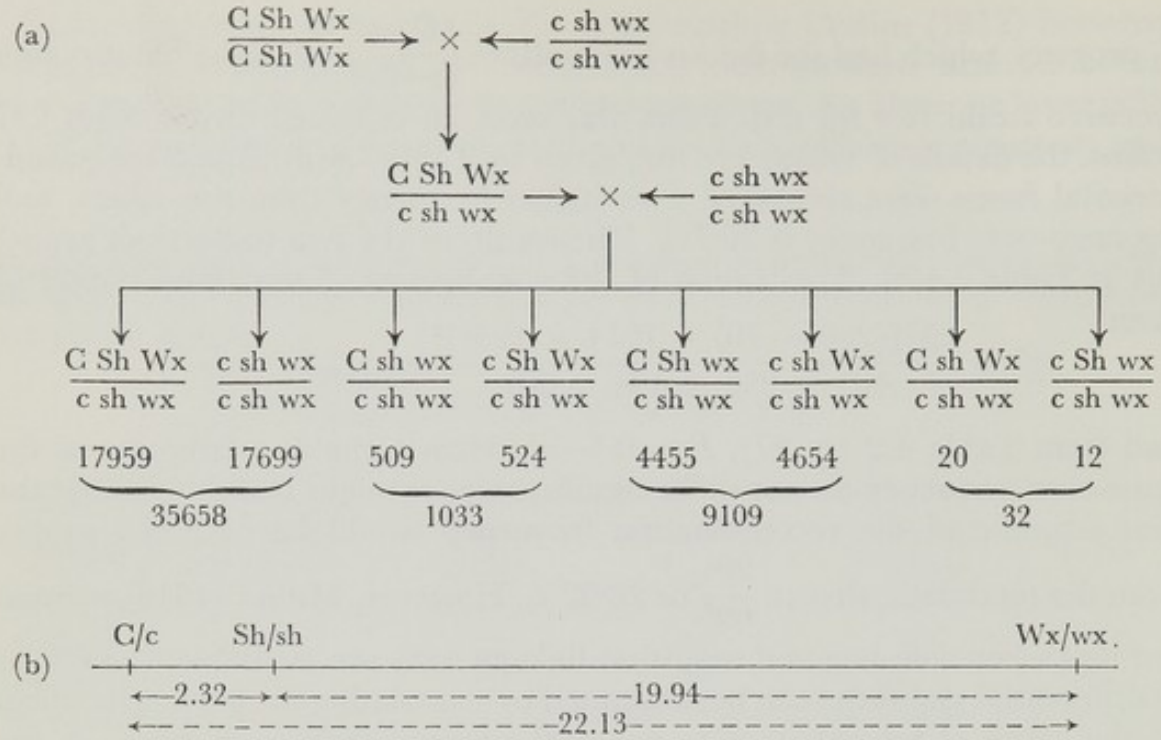


FIGURE 7.4 (a) Numbers of progeny of various genotypes from a *Zea* test-cross (Stadler 1926).
C = Dominant gene for Coloured aleurone layer of the endosperm.
c = Recessive allele for colourless aleurone.
Sh = Dominant gene for normal Non-shrunken endosperm.
sh = Recessive allele for shrunken endosperm.
Wx = Dominant gene for Starchy (translucent) endosperm.
wx = Recessive allele for waxy (opaque) endosperm.
(b) Linkage map based on the data in (a).

example discussed in § 7.4, the slight discrepancy in the figures is due to double cross-overs, since

$$p + q = r + \frac{64}{45832}.$$

If another gene *X/x* were available between *Sh/sh* and *Wx/wx*, the recombination frequency of *Sh/sh* and *X/x* plus the recombination frequency of *X/x* and *Wx/wx* would no doubt exceed 19·94%.

The frequency of recombination between *C/c* and *Wx/wx* in Stadler's experiment (22·1%) was significantly less than that found by Bregger (28·6%). Such variation in recombination frequencies is of frequent occurrence, and may be due to genetic or to environmental factors affecting the frequency of crossing-over. It is evident that distances on linkage maps have no absolute values, but are likely to differ in different experiments.

§ 7.6 Evidence that crossing-over is associated with chromosomal exchange

Creighton and McClintock (1931) were the first to obtain convincing evidence that genetic crossing-over of linked characters is associated with exchange of parts between homologous chromosomes. They bred a strain

of *Zea mays* heterozygous for two peculiarities of chromosome 9 and for two hereditary characters known to be carried by this chromosome. The chromosomal characters were the presence or absence of a knob at the end of the short arm, and the presence or absence of a part of chromosome 8 in place of part of the long arm. The genetic characters were two of those referred to in § 7.5, namely, a Coloured (*C*) or colourless (*c*) aleurone layer to the endosperm of the maize grain, known to be due to a gene difference located near the knob, and Starchy (*Wx*) or waxy (*wx*) endosperm, due to a factor difference located near the point of attachment of the portion of chromosome 8. The combinations of these characters in the heterozygote were as follows:

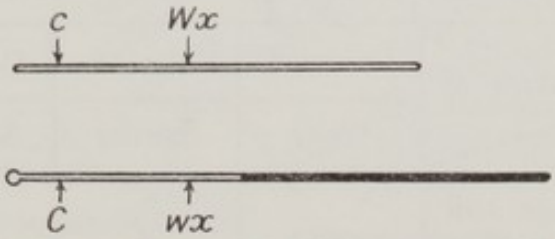


FIGURE 7.5 The pair of no. 9 chromosomes in Creighton and McClintock's strain of *Zea mays* heterozygous for two structural characters of the chromosome and for two endosperm characters. The structural characters were the presence and absence at one end of the chromosome of a knob, and at the other end of an interchange of a segment with a segment (shown black) of chromosome no. 8. C = Gene for Coloured, *c* = allele for colourless aleurone layer to the endosperm. *Wx* = Gene for Normal Starchy endosperm, *wx* = allele for waxy endosperm.

On one edition of chromosome 9 there was the knob, the dominant gene *C* for Coloured aleurone, the recessive gene *wx* for waxy endosperm, and the portion of chromosome 8. On the other edition of chromosome 9, the other alternative of each of these characters was present (Fig. 7.5). This quadruple heterozygote was crossed with a plant having no knobs and no parts of chromosome 8 on either of its 9th chromosomes, and also homozygous for the recessive gene *c* for colourless aleurone, but heterozygous for the Starchy-waxy character-difference. There were 27 progeny, of which 13 could be fully scored and interpreted (see Table 7.5). Since both parents were heterozygous for the Starchy-waxy character-difference, any progeny similarly heterozygous have been omitted from the table since it cannot be established from which parent the *Wx* and *wx* factors were derived. (As indicated in Chapter 5, the *Wx Wx* and *Wx wx* genotypes can be distinguished by examination of the pollen after staining with iodine.) All 13 progeny showed complete linkage between the aleurone colour factors and the knobbed or knobless condition, and 11 of them also showed complete linkage between the quality of the endosperm and presence or absence of the interchange. When the genetical characters crossed-over, so did the cytological ones. In two of the progeny there was evidently a cross-over between the *Wx*/*wx* locus and that of the point of attachment of the part of

chromosome 8. The data form striking evidence that genetical crossing-over is associated with exchange of parts between homologous chromosomes. Whether this comes about by breakage and rejoining, or in the process of replication of the hereditary material, is discussed in Chapter 16.

TABLE 7.5 Numbers of progeny found by Creighton and McClintock (1931) from a *Zea* cross, using as one parent a plant heterozygous both for two abnormalities in chromosome 9 and for two characters of the endosperm (aleurone colour and food reserve) known to be due to genes borne by this chromosome. A normal chromosome 9 was present in addition to the one described in the table. Individuals heterozygous for the food reserve characters have been omitted.

Chromosome 9 Characters / Endosperm Characters		Coloured	Colourless	Coloured	Colourless
		Waxy	Starchy	Starchy	Waxy
Knob present	Interchange with chromosome 8 present	3	0	0	0
No knob	No interchange	0	5	0	0
Knob present	No interchange	0	0	1	0
No knob	Interchange with chromosome 8 present	0	2	0	2

A few weeks later Stern (1931) reported similar results for *Drosophila melanogaster*. He had obtained four different strains of female flies, each heterozygous for two abnormalities in the *X*-chromosome and for two sex-linked characters. Over 27,000 progeny from these females were scored for the latter characters, and 364 of them were also examined cytologically and scored for the chromosomal characters. All four experiments gave similar results, but only one will be described.

The female parents in this experiment carried the factor for the recessive character carnation-coloured eyes on one of their *X*-chromosomes, which was structurally normal. On the other *X*-chromosome was the normal allele of carnation eye and the dominant gene for Bar eye. This chromosome was also structurally abnormal in two respects: firstly, it had part of a *Y*-chromosome attached at one end just beyond the carnation locus; secondly, it was broken into two parts, the break being just beyond the Bar locus. This means that the proximal part carried both the Bar gene and the normal allele of carnation. The distal part was attached to the small fourth chromosome (Fig. 7.6). The male parent was structurally normal, but carried the gene for carnation eyes on its *X*-chromosome. Details of the 8231 progeny of this cross are

given in Table 7.6. Out of this number 107 were examined cytologically, including 54 in which crossing-over had occurred between the loci for carnation and Bar. Without exception, these 54 flies also showed crossing-over for the chromosomal characters. Bar eye-shape was always associated

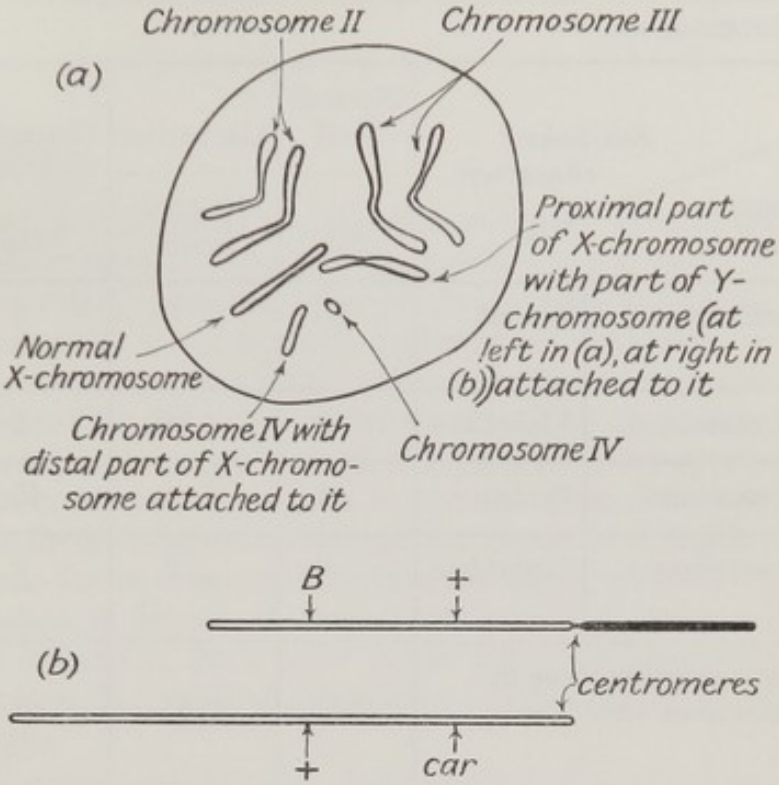


FIGURE 7.6 The chromosomes of the female parents in one of Stern's experiments with *Drosophila melanogaster* which demonstrate that genetical crossing-over is associated with physical exchange between the corresponding homologous chromosomes.
(*a*) Polar view of mitotic metaphase.
(*b*) The *X*-chromosomes. *B* = Dominant gene for Bar eye-shape, and the plus sign (+) at the corresponding position = recessive normal allele; *car* = recessive gene for carnation eye-colour, and the plus sign at the corresponding position = dominant normal allele. The attached part of the *Y*-chromosome is shown black.

with the broken *X*-chromosome to which it was closely linked, and normal Red eye-colour was associated with the attached piece of *Y*-chromosome. Indeed, as indicated in the table, there was only one fly out of the 107 which showed breakdown of these linkages: a cross-over had apparently occurred between the carnation locus and the point of attachment of the *Y*-chromosome segment.

TABLE 7.6 Numbers of progeny found by Stern (1931) from a *Drosophila* cross, using females heterozygous both for two abnormalities in the *X*-chromosome and for two sex-linked characters, carnation eye-colour and Bar eye-shape. A normal *X*-chromosome (in females) or *Y*-chromosome (in males) was present in addition to the *X*-chromosome described in the table. The shortage of normal progeny (Red round eyes) is thought to be due to their failure to survive owing to loss of one of their small fourth chromosomes.

X-chromosome characters / Sex-linked characters		Normal Red	Carnation	Carnation	Normal Red
		Bar	Normal round	Bar	Normal round
Part of *Y*-chromosome attached	Broken	26	0	0	0
Without *Y*-chromosome	Unbroken	0	26	0	0
Without *Y*-chromosome	Broken	1	0	45	0
Part of *Y*-chromosome attached	Unbroken	0	0	0	9
Total progeny (including those in which chromosomes were not examined)		4001	4157	61	12

Stern's data constitute further evidence that genetical crossing-over involves exchange of material between the appropriate homologous chromosomes. Thus, just 20 years after Morgan's crucial discovery of new combinations of two sex-linked character-differences in the progeny of a *Drosophila* cross, which led him to postulate interchange of parts between homologous chromosomes, Stern, using both genetical and cytological characters in the identical chromosome, obtained overwhelming evidence of the truth of Morgan's postulate. The mechanism of crossing-over is discussed in Chapter 16.

8. *The chiasmatype theory*

§ 8.1 Chiasmata

As indicated in Chapter 6, the chromosome pairs seen at late prophase I of meiosis usually present the appearance either of a cross or of one or more loops. Janssens (1909) gave the name *chiasma* (meaning a cross) to the nodes between the arms or loops in these diplotene or diakinetic configurations. He also adopted in the same publication a particular hypothesis (described below) to account for the origin of these nodes. The use of the term chiasma was at first intimately bound up with this particular interpretation of them. However it is customary nowadays to use the term for these nodes, irrespective of the theory adopted to account for them. It is highly inconvenient to have a name for an observed structure the use of which is dependent on a theory to explain it. Hence, throughout this book chiasma will be used as Janssens first defined it, for a node in the paired chromosomes at the first division of meiosis. It should not be taken to imply any particular interpretation of its origin.

§ 8.2 Janssens' theory

At the diplotene stage the chromosomes have already divided into two daughter-chromatids, with the result that four threads are present in each chromosome-pair. Janssens had studied meiosis in several organisms, but particularly at sperm-formation in the amphibian *Batrachoseps attenuatus*. At the chiasmata at the diplotene stage, whenever the fixation and staining had been particularly good, he observed that of the four filaments, two cross each other and two do not (Fig. 8.1(*a*) and Pl. 3(*a*)). At anaphase I, when the chiasmata break down, the two chromatids which pass to one pole of the spindle were seen to lie together on one side of each chiasma and to lie apart on the other side (Fig. 8.1(*c*) and (*d*)). It was evident that two of the four chromatids crossed at each chiasma. These observations have been amply confirmed in many organisms.

Janssens believed that prior to the formation of the chiasmata, the paternal and maternal chromosomes in the early diplotene stage were loosely coiled round each other, as in Fig. 8.2(*b*). In order to derive chiasmata from such a loose coil, Janssens postulated that the paternal and maternal chromatids made contact at intervals, and that one of each penetrated the other until they broke, whereupon they rejoined in new ways paternal to maternal, and *vice versa*. The other two chromatids (one paternal and one maternal) were considered to remain intact, so giving rise to the typical chiasma with two

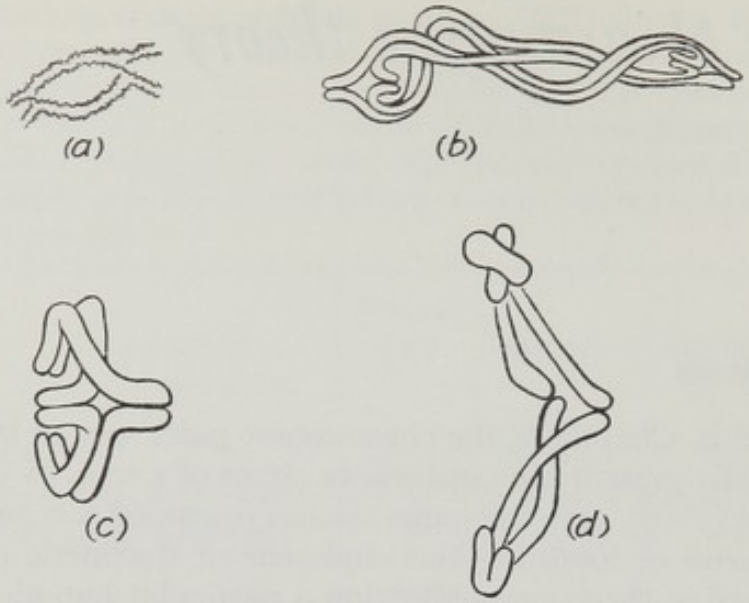


FIGURE 8.1 Chromosome-pairs showing chiasmata at meiosis in males of *Batrachoseps attenuatus* (from Janssens, 1909). The number and position of the chiasmata varies from cell to cell. (*a*) Diplotene stage (2 chiasmata). (*b*) Diakinesis (3 chiasmata). (*c*) Metaphase I (1 chiasma). (*d*) Anashase I.

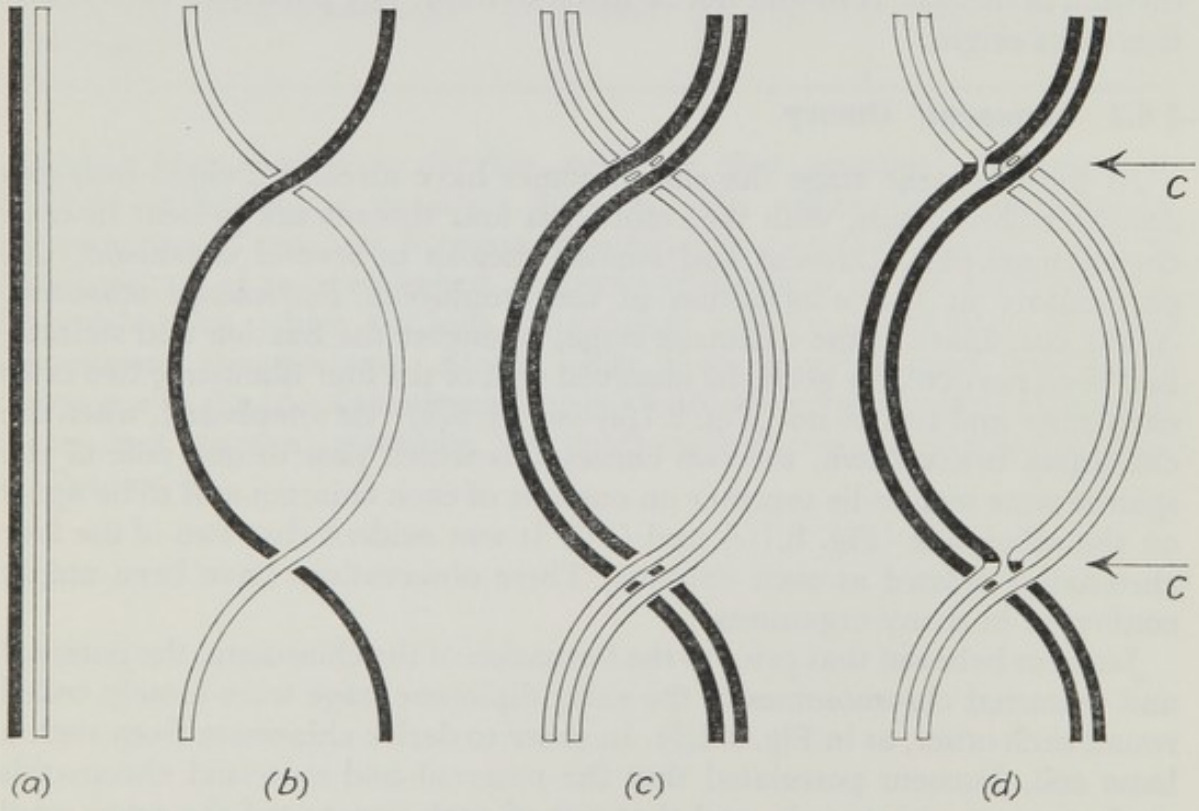


FIGURE 8.2 Diagrams to show the structure of a pair of homologous chromosomes at (*a*) the pachytene stage and (*b–d*) successives early diplotene stages, according to Janssens' hypothesis of the origin of chiasmata *C* = Chiasma. Black = paternal and white = maternal origin.

chromatids crossing each other and two not (Fig. 8.2(*d*)). This remarkable hypothesis has been a centre of controversy ever since it was first put forward.

Janssens also used the teleological argument that the occurrence of his hypothetical exchanges between one paternal and one maternal chromatid at each chiasma would ensure that the four nuclei, to which the four chromatids of each diplotene chromsome-pair were distributed, had different combinations of paternal and maternal segments of each chromosome, and hence would account for the universal existence of two nuclear divisions in meiosis and four products. He argued that meiosis was a mechanism for the production of four haploid nuclei all of different genotypes. He pointed out that there would then be segregation of some factor differences at each division of meiosis (Fig. 8.3(*b*)) in contrast to the widely held view that segregation of paternal from maternal chromosomes occurred at the heterotype or first division of meiosis (Fig. 8.3(*a*)), with the identical daughter-chromosomes

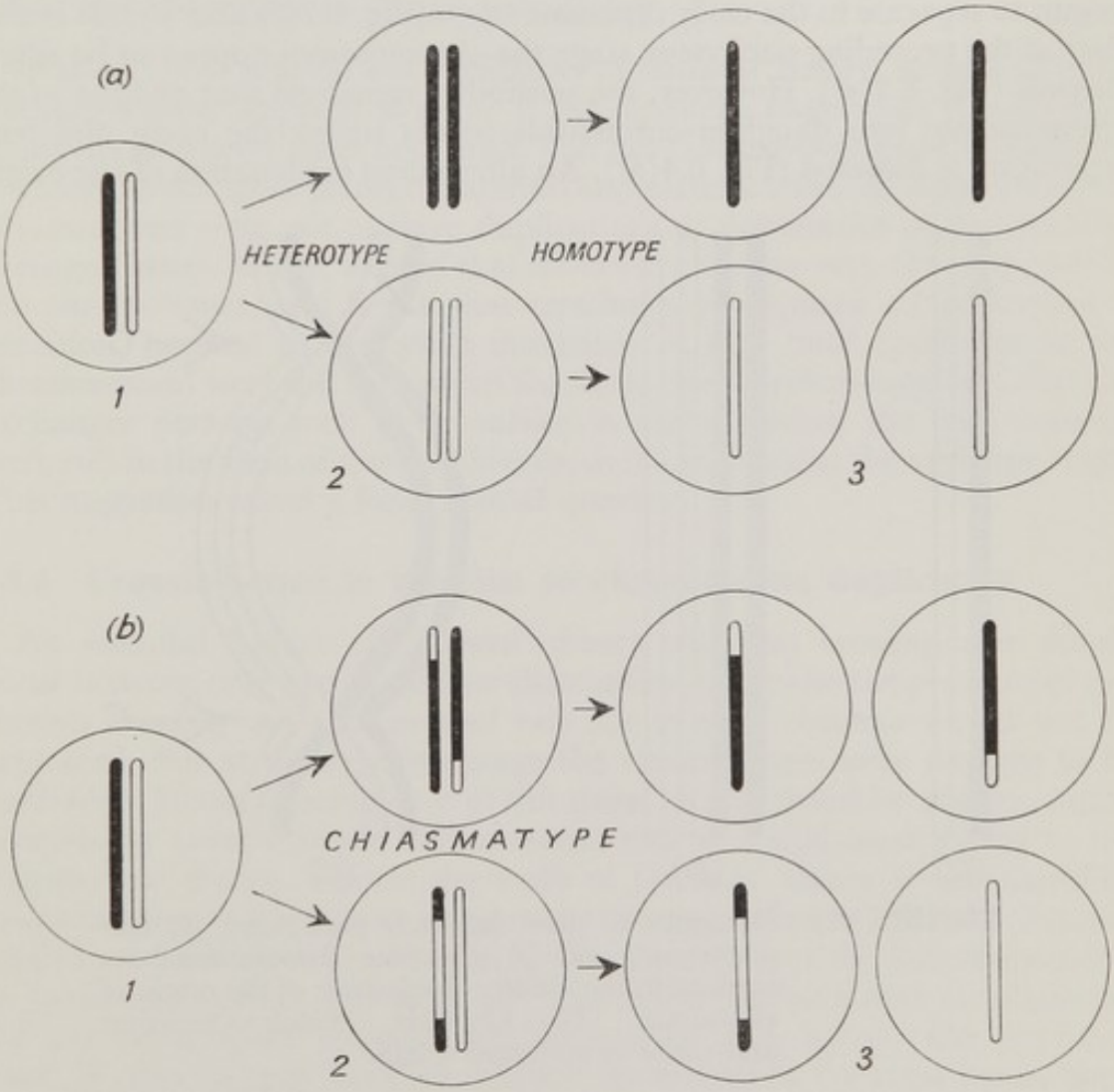


FIGURE 8.3 Diagrams of (1) the pachytene stage of prophase I, (2) telophase I, and (3) telophase II, according to (*a*) the hypothesis that meiotic segregation occurs only at the first division, and (*b*) Janssens' hypothesis that it occurs partly at each division. Black = paternal and white = maternal origin.

composing each of them separating from one another at the homotype or second division (Fig. 8.3(*a*)). To emphasise this difference from the heterotype-homotype theory of meiotic segregation, he called his idea the *chiasmatype* theory.

Janssens also pointed out that his theory provided an escape from the dilemma of the existence of a larger number of freely assorting allelomorphic characters than there are pairs of chromosomes.

§ 8.3 The classical theory

The weak link in Janssens' argument was his postulation that loose coils preceded the formation of chiasmata. Since he was observing fixed and stained material, it was difficult to test this postulate by observation. It was based on the assumption that division of each chromosome into two chromatids did not occur until after the paternal and maternal chromosomes had begun to separate in the early diplotene stage (Fig. 8.2(*b*) and (*c*)). It is true that at the preceding pachytene stage the chromosomes appear to be single threads (Fig. 8.2(*a*)). However, the possibility remained that division of the chromosomes into daughter-chromatids occurs before the early diplotene separation is initiated (Fig. 8.4(*b*)). An alternative explanation of the origin

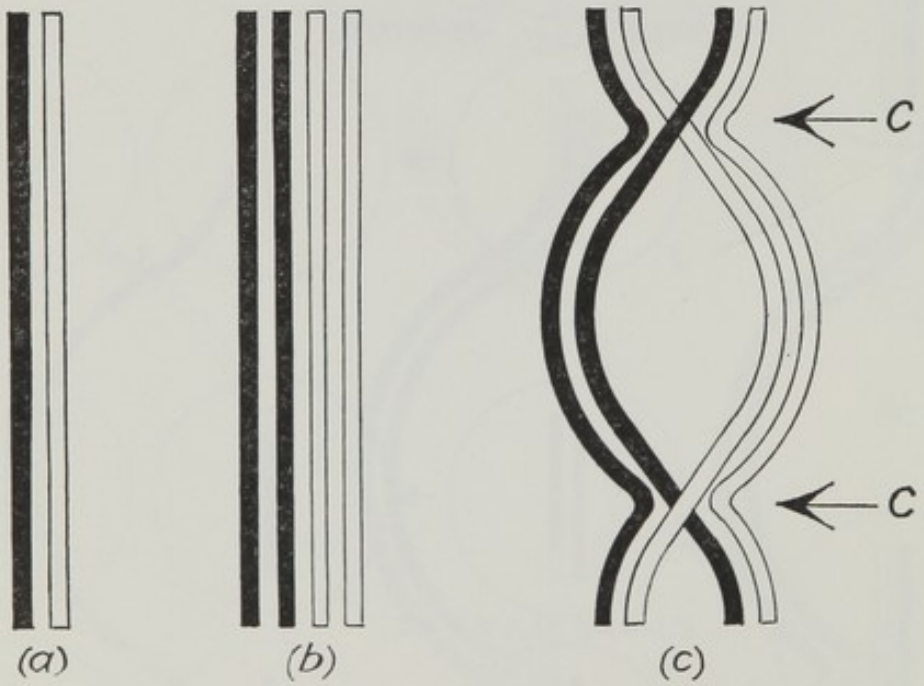


FIGURE 8.4 Diagrams to show (*a*) early pachytene, (*b*) late pachytene, and (*c*) diplotene chromosomes according to the classical hypothesis of the origin of chiasmata. C = Chiasma. Black = paternal and white = maternal origin.

of chiasmata is then possible, namely that at a chiasma there is a change of pairing partner. At one region of the chromosome two paternal chromatids would be associated and likewise the two maternal ones, but on the other side of a chiasma each pair of chromatids would consist of one paternal and one

maternal thread (Fig. 8.4(*c*)). This *classical hypothesis* to explain chiasmata was preferred by most cytologists becaust it did not require breakage and rejoining of chromosomes, with consequent threat to their permanence and individuality.

§ 8.4 Genetic evidence in support of Janssens' theory

When Morgan (1911*a*) first discovered that new combinations of sex-linked characters were appearing in the F_2 generation of a *Drosophila* cross, he favoured De Vries' theory of factor exchange. However, shortly afterwards he realised that the sex-linked factors were not assorting at random, as De Vries' theory demanded, but (as indicated in the last chapter) were showing partial linkage of various intensities. He then favoured Janssens' chiasmatype theory as an explanation.

§ 8.5 An objection to Janssens' theory

Muller (1916) pointed out a difficulty of Janssens' theory, namely that the exchanges were supposed to occur at the diplotene stage when the chromosomes are relatively short and thick, and yet crossing-over must be between precisely homologous points of paternal and maternal threads if the cross-over chromosomes were not to have duplications or deficiencies of the linearly-arranged genes. Muller argued that factors must be set very close together in the chromosomes, and hence that crossing-over required a high degree of precision, because in *Drosophila* mutations in new 'loci' (positions in the chromosomes) were still turning up regularly. He therefore suggested that the exchanges perhaps took place earlier in meiosis when the chromosomes were still in the form of slender threads, as for example at the zygotene stage. This suggestion raised a fundamental question.

§ 8.6 Crossing-over in relation to chromosome duplication

An essential feature of Janssens' theory was that crossing-over should occur between only two of the four chromatids, otherwise the presence of two threads crossing one another and two not at each chiasma would not be explained. But at the zygotene stage the chromosomes were thought to be undivided. Hence crossing-over at this stage, as postulated by Muller, would presumably involve whole chromosomes and so would be contrary to the chiasmatype theory. To test the truth of Janssens' theory it was therefore essential to know whether crossing-over occurred before or after the chromosomes had duplicated. If exchange involved only two of the four chromatids, as Janssens supposed, this would be conclusive evidence that crossing-over did not occur before chromosome duplication (Fig. 8.5(*a*)). On the other hand, if crossing-over involved all four chromatids at the same locus, there were two possible explanations: either exchange occurred while the chromosomes were still single threads and the subsequent duplication produced two editions of each (Fig. 8.5(*b*)); or all four chromatids were broken at corresponding positions and were subsequently joined paternal to maternal,

and *vice versa* (Fig. 8.5(*c*)). Janssens himself thought he had cytological evidence that the latter alternative occurred occasionally.

Bridges (1916) was the first to obtain information on this fundamental question of whether crossing-over takes place before or after chromosome duplication. During the course of his work with the *XXY* strain of *Drosophila*

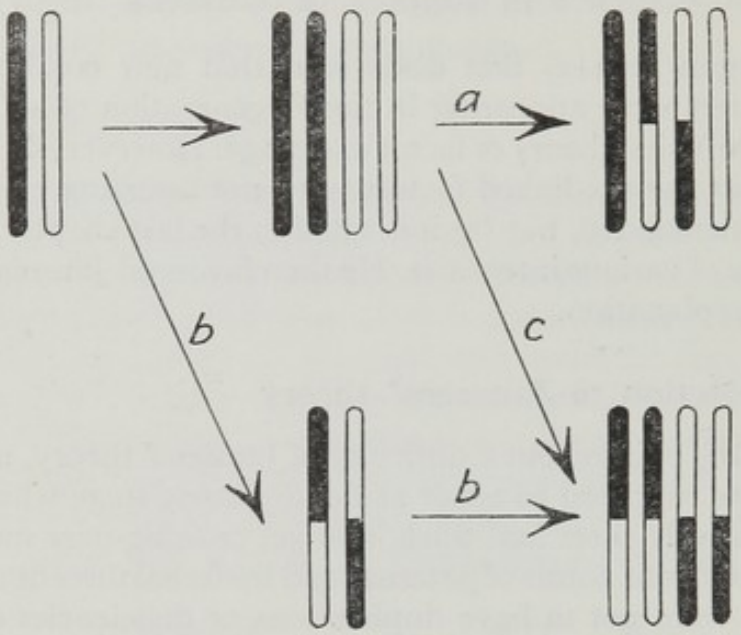
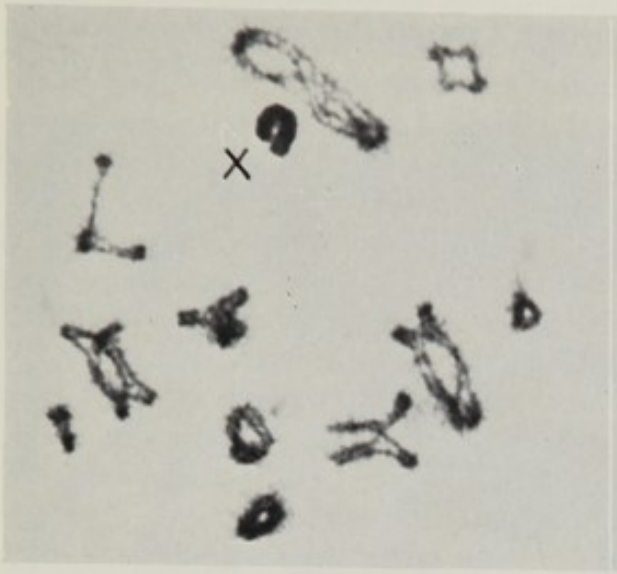


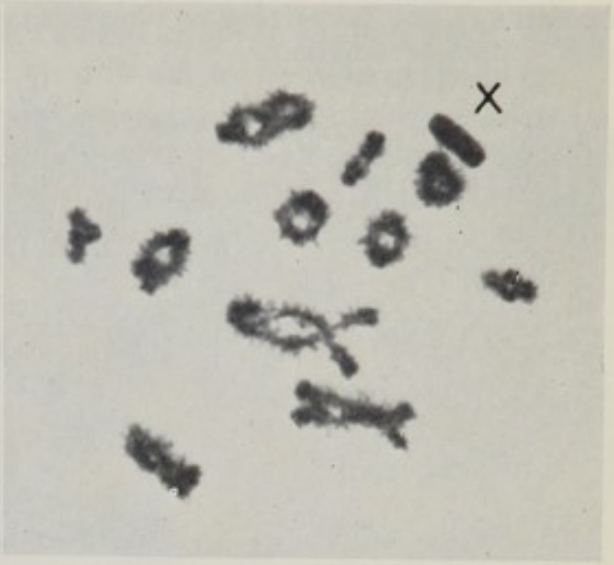
FIGURE 8.5 Possible ways in which crossing-over might occur.
(*a*) Janssens' chiasmatype hypothesis: crossing-over after chromosome duplication and involving only two of the four chromatids.
(*b*) Crossing-over at the two-strand stage before chromosome duplication.
(*c*) Crossing-over after chromosome duplication but involving all four chromatids at the same locus (thought by Janssens to occur occasionally).
Black = paternal and white = maternal origin.

melanogaster (see Chapter 6), he obtained *XXY* females which were heterozygous for the sex-linked characters vermilion eye-colour and eosin eye-colour. These characters are both recessive to the normal Red eyes and are due to genes showing about 30% recombination with one another. The doubly-heterozygous *XXY* females, some with vermilion and eosin in coupling and some with them in repulsion, were crossed with Bar-eyed but otherwise normal males. The dominant gene Bar is sex-linked and leads to the production of a narrow eye instead of the normal round eye. Any progeny from this cross which receive an *X*-chromosome from their father will have Bar eyes. Hence any female progeny with eyes of the normal round shape must have received both their *X*-chromosomes from their mother. They will correspond to the flies in class (i) of Table 6.2, which represent about 2% of the progeny. From the coupling crosses, 422 non-Bar females

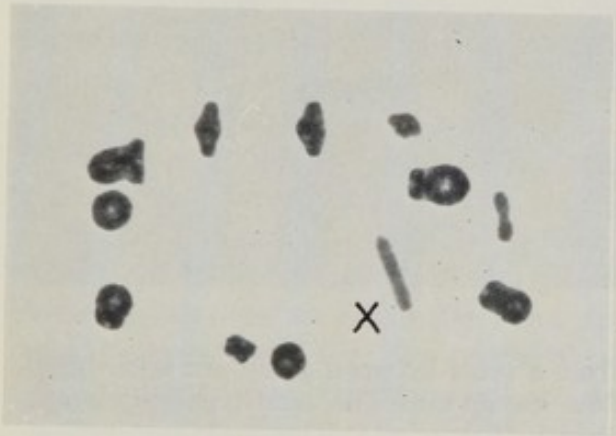
PLATE 3 Meiosis in acetic-orcein squash preparations of male *Schistocerca gregaria* ($2n = 22 + X$) (continued from Plate 2). Magnification ca. ×1000. X = X-chromosome. In (*a*) and (*b*) the autosomal bivalents (but not the X-chromosome) have a lampbrush appearance (see Chapters 17 and 18).
(*Courtesy of S. A. Henderson*)



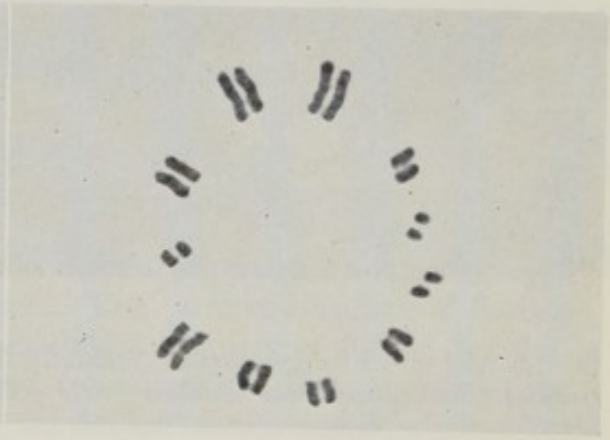
(*a*) Diplotene



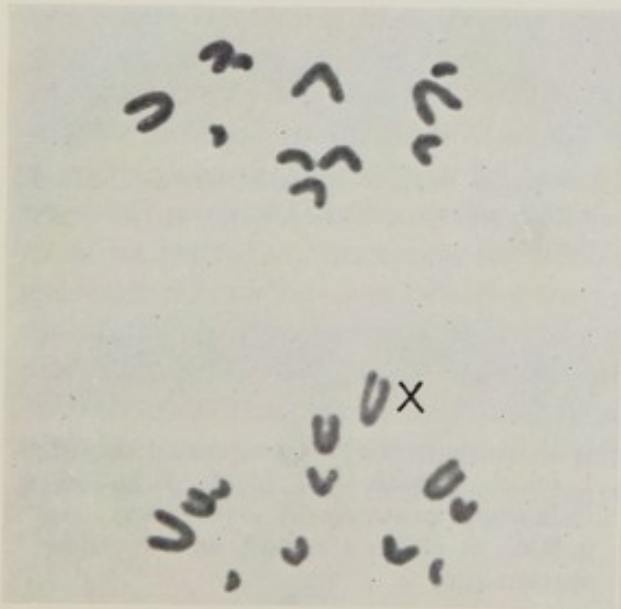
(*b*) Diakinesis



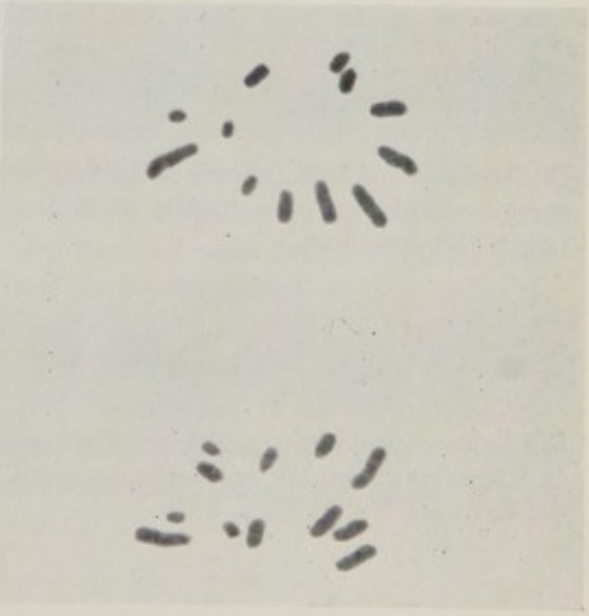
(*c*) Metaphase I



(*e*) One cell at metaphase II (polar view)



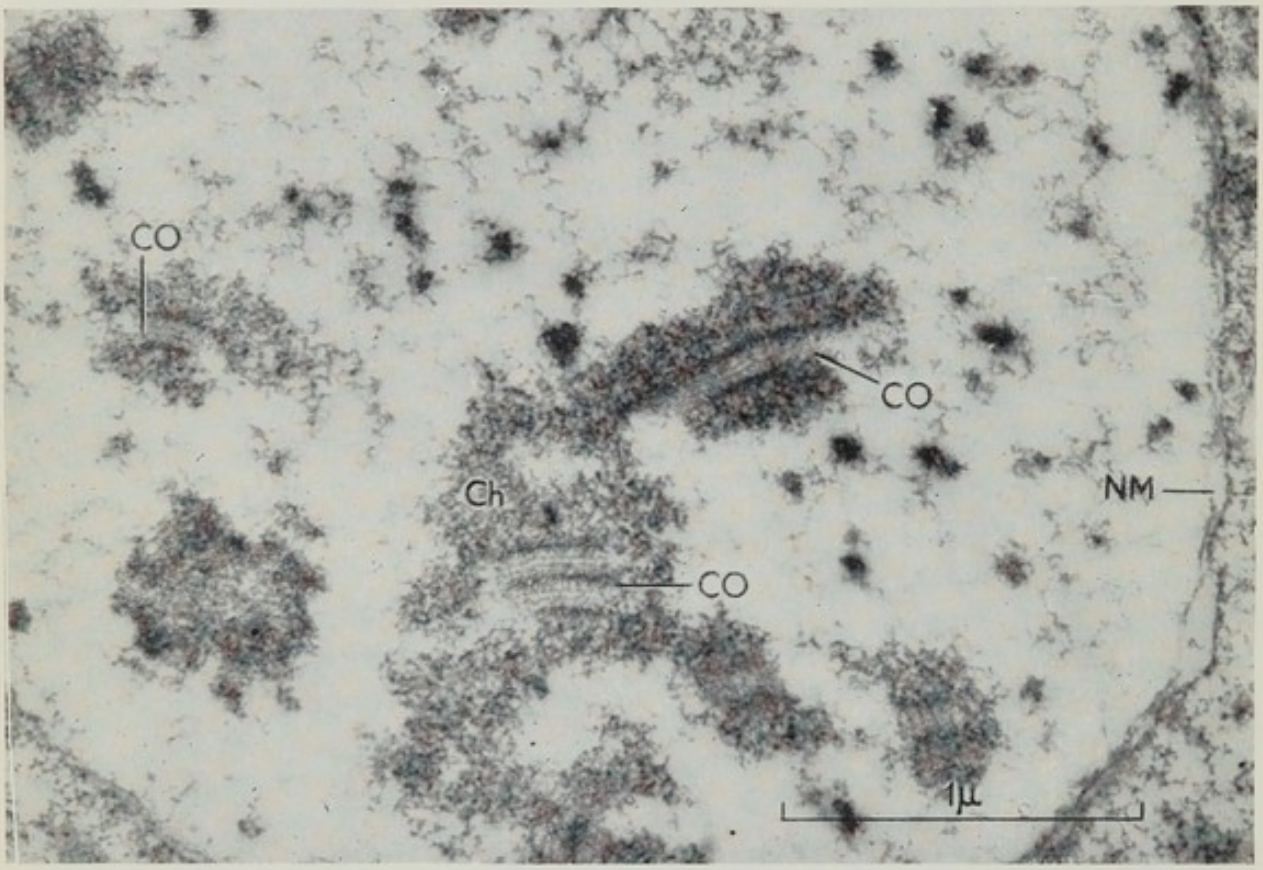
(*d*) Anaphase I



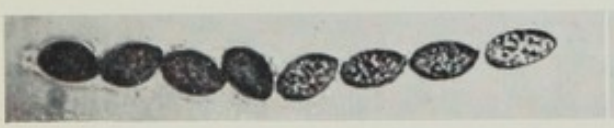
(*f*) One cell at anaphase II (side view)

PLATE 4

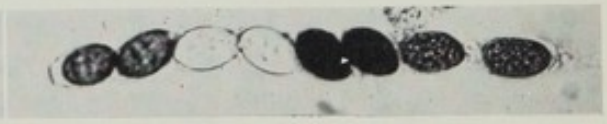
(*a*) Electron micrograph of a thin section of an oocyte nucleus of the Dipteran insect *Chironomus tentans* at pachytene of meiosis, showing synaptinemal complexes (see p. 131). Slightly hypotonic fixation has been used to bring out the structural components of the complexes. Magnification ×35,000. *Ch* = chromosomal fibrils of bivalents. *Co* = complex, consisting of 2 lateral elements, 2 much finer central longitudinal elements, and numerous exceedingly fine fibres crossing the pairing space between the longitudinal elements. *NM* = nuclear membrane. (*Courtesy of G. Meyer*)



(*b*)–(*e*) Asci of the fungus *Sordaria brevicollis* from a cross between a mutant with buff-coloured ascospores and another with yellow ascospores. The wild-type spores are black and the double-mutant spores are white (colourless). The buff and yellow mutants show linkage with about 9% recombination. Magnification ca. ×350. (*Courtesy of M. H. V. Cooray*)



(*b*) Ascus with 4 buff (to left) and 4 yellow spores, implying no crossing-over between the gene loci.



(*c*) Ascus with 2 buff, 2 white, 2 black and 2 yellow spores (1 crossover between the loci).

(*d*) Ascus with 4 white and 4 black spores (a four-strand double crossover between the loci: see Fig. 8.12, p. 110).

(*e*) Ascus with the spore sequence: 3 buff, 1 white, 1 yellow, 1 black, 2 yellow; indicating postmeiotic segregation (see p. 306) at either the buff or the yellow mutant site.

were obtained, of which 414 had Red eyes like their mother and 8 had vermilion, eosin or vermilion-eosin eyes. Similarly, from the repulsion crosses there were 168 non-Bar females with Red eyes and 5 with vermilion or eosin eyes. The genotypes of the vermilion- and eosin-eyed females were established by further breeding and the results are given in Table 8.1. All those that were homozygous for eosin were found to be heterozygous for vermilion, and *vice versa*. To obtain flies of these constitutions requires crossing-over between the two *X*-chromosomes of the mother during maturation of her eggs, and furthermore such crossing-over must take place between individual chromatids and involve only two of them (one derived from each *X*-chromosome) (see Fig. 8.6). With no crossing-over, or with crossing-over before chromosome duplication, any two of the four chromatids will always be either identical or complementary, that is of genotypes (i) to (vi) in Fig. 8.6. The occurrence of genotypes (viii) to (x) was proof of crossing-over after chromosome duplication. Altogether, Bridges obtained information about 11 cross-overs between the loci of vermilion and eosin and every one of them was between two chromatids.

Anderson (1925) obtained more evidence that crossing-over occurred only at the four-strand stage. A female *Drosophila* was discovered after treatment with X-rays, in which the two *X*-chromosomes were joined together at one end. This union was demonstrated cytologically, for a single V-shaped chromosome replaced the two rod-shaped *X*-chromosomes. It was also shown by the fact that on crossing with a normal male, sex-linked characters which she possessed were manifest in her daughters and not in her sons. This was in direct contrast to the usual behaviour of sex-linked characters, which show criss-cross inheritance (see Chapter 6). The daughters of the attached-*X* female will inherit her two joined *X*-chromosomes, and moreover there will be opportunity at meiosis during maturation of her eggs for crossing-over to occur between them. The parent attached-*X* female was heterozygous for the recessive sex-linked characters forked bristles, garnet eye-colour, tan body-colour, and cut wing-tips. If no crossing-over occurred the daughters would have a double *X*-chromosome identical with that of their mother. On the other hand, crossing-over in certain intervals of the chromosome could lead to one or more of the recessive characters becoming homozygous and hence manifest, just as in Bridges' earlier work.

Out of 4344 daughters of attached-*X* females heterozygous for these genes, Anderson found totals of 226 (5·2%) with forked bristles, 414 (9·5%) with garnet-coloured eyes, 698 (16·1%) with tan-coloured bodies and 672 (15·5%) with cut wings. The occurrence of these individuals, homozygous for one or more recessive characters for which the mother was heterozygous, was evidence of crossing-over between the joined *X*-chromosomes, and in order to obtain more information, a random sample of 188 of the daughters was subjected to detailed study. The genetic constitution of each *X*-chromosome of each individual was established from the frequencies of different phenotypes in their progeny from suitable crosses. The results are set out in Table 8.1 with the four pairs of alleles taken two at a time. In each instance, of the

TABLE 8.1 Numbers of different genotypes of female *Drosophila melanogaster* when the two *X*-chromosomes of each individual have both been derived from their mother. Bridges' (1916) data refer to *XXY* flies, Anderson's (1925) to attached *XX*, and Bridges & Anderson's (1925) to triploid *XXX*. A dash (—) as distinct from zero (0) indicates flies of normal phenotype, the genotype of which was not determined. There were 414 of these in the coupling crosses (column 2) and 168 in the repulsion crosses (column 3).

The Ten Possible Genotypes (Female Parent $\frac{AB}{ab}$)	*XXY* Bridges (1916)		Attached *XX* Anderson (1925)			Triploid *XXX* Bridges & Anderson (1925)			
	Vermilion (*a*) and eosin (*b*)	Vermilion (*a*) and eosin (*B*)	Forked (*a*) and garnet (*B*)	Garnet (*A*) & tan (*b*)	Tan (*a*) and cut (*B*)	Bar or forked and dusky or miniature	Dusky or miniature and lozenge or tan	Lozenge or tan and ruby or bifid	Ruby or bifid and scute or yellow
(i) $\frac{AB}{AB}$	—	0	8	15	21	2	3	3	5
(ii) $\frac{ab}{ab}$	2	0	11	25	36				
(iii) $\frac{AB}{ab}$	—	—	126	103	104	153	167	140	126
(iv) $\frac{Ab}{aB}$	—	—	11	9	8	3	1	2	0
(v) $\frac{Ab}{Ab}$	0	—	0	0	0	0	0	0	0
(vi) $\frac{aB}{aB}$	0	0	0	0	0	0	0	0	0
(vii) $\frac{AB}{Ab}$	—	—	2	3	7	0	0	2	5
(viii) $\frac{aB}{ab}$	3	1	1	5	4				
(ix) $\frac{AB}{aB}$	—	4	10	13	3	1	2	7	16
(x) $\frac{Ab}{ab}$	3	—	19	15	5				

10 genotypes theoretically possible, 8 were represented. The missing ones were always those with two identical cross-over chromosomes (genotypes (v) and (vi)) and they can only be obtained by postulating that crossing-over occurs at the two-strand stage (unless double cross-overs are invoked) (see Fig. 8.6). Conversely there were present all the genotypes with one

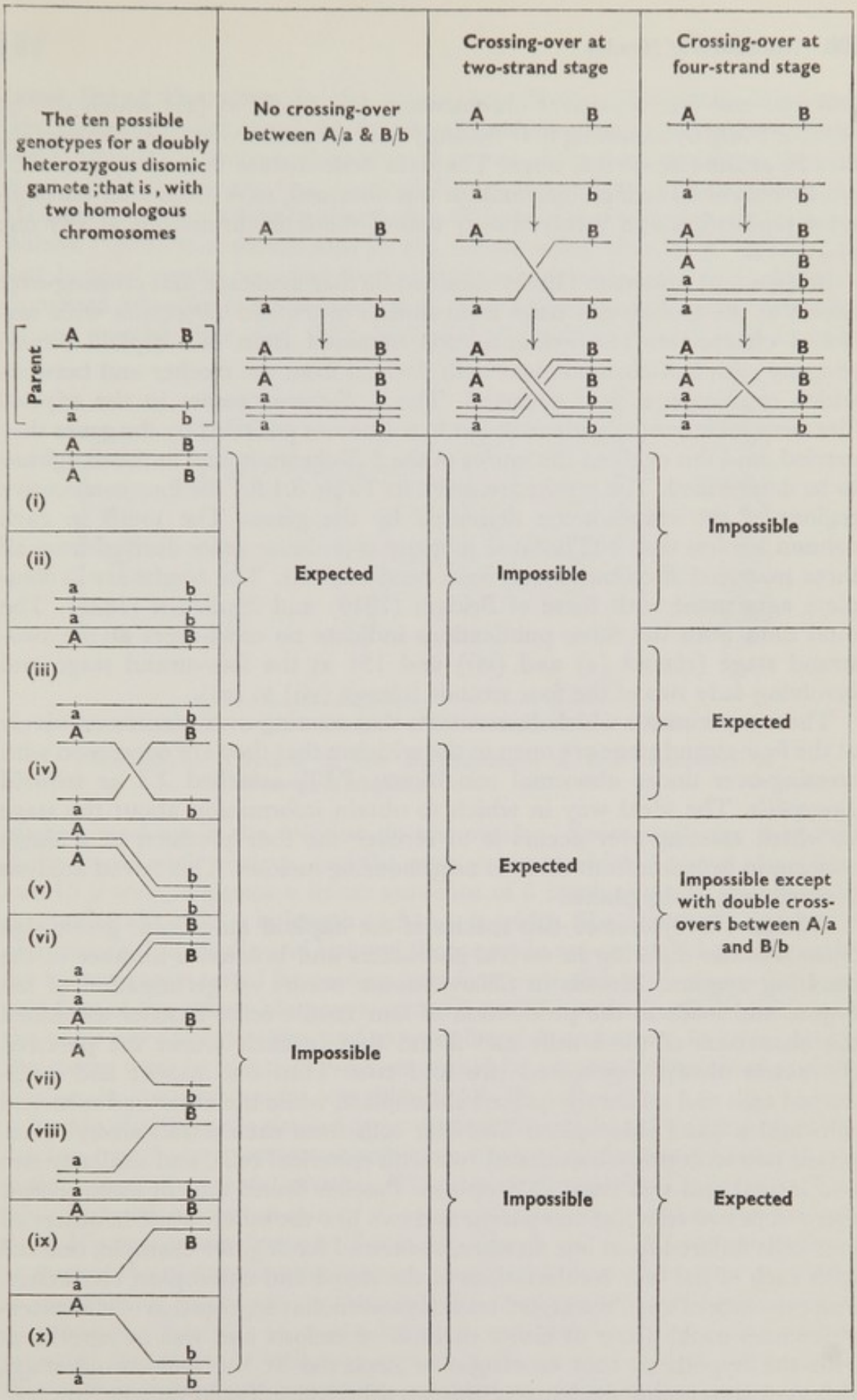


FIGURE 8.6 The genotypes expected for the gametes from a double heterozygote $\frac{AB}{ab}$ if two of the four meiotic chromatids pass into each gamete. The expectations with no crossing-over, with crossing-over at the two-strand stage, and with crossing-over at the four-strand stage, are shown in separate columns.

parental and one cross-over chromosome (nos. (vii) to (x)) which can be obtained only by assuming that crossing-over occurs between two chromatids, that is, at the four-strand stage. The data demonstrate that every one of 87 cross-overs, about which information was obtained, took place after chromosome replication and involved only two of the four chromatids at any one place.

Bridges and Anderson (1925) obtained further evidence that crossing-over occurs at the four-strand stage from studies of triploid *Drosophila* with sex-linked characters. The evidence was obtained from 182 diploid female progeny with 2 *X*-chromosomes both derived from the mother and between which crossing-over had occurred. The 3 *X*-chromosomes in the triploid flies were each uniquely identified at five different positions by the genes they carried, and this enabled the source of the 2 *X*-chromosomes in the daughters to be determined. The results are given in Table 8.1 for the four consecutive regions of the chromosome delimited by the genes. The totals in each column are less than 182 because progeny containing genes derived from all three maternal *X*-chromosomes have been omitted. The results are in complete agreement with those of Bridges (1916) and Anderson (1925). The total data from the three publications indicate no cross-overs at the two-strand stage (classes (v) and (vi)) and 131 at the four-strand stage and involving only two of the four strands (classes (vii) to (x)).

These experiments which demonstrate that crossing-over occurs exclusively at the four-strand stage are open to the criticism that they are concerned with crossing-over under abnormal conditions: *XXY*, attached *XX* or triploid *Drosophila*. The ideal way in which to obtain information about the stage at which crossing-over occurs is to recover the four products of a single meiosis, in isolation from those of neighbouring meioses. This tetrad analysis is possible in many plants.

Pascher (1918) crossed two species of the haploid unicellular green alga *Chlamydomonas* differing in several characters and isolated a number of the resulting zygotes. Meiosis in *Chlamydomonas* occurs on germination of the zygote and leads to the production of four motile cells. Pascher examined the characters of these cells and found that in each tetrad the parental characters always segregated two and two. Thus one parent had pear-shaped cells and a laterally-placed chloroplast, while the other had spherical cells and a basal chloroplast. The four cells from each tetrad always comprised two with pear-shaped and two with spherical cells, and similarly two had lateral and two basal chloroplasts. Pascher found that in some tetrads there were two cells like one parent and two like the other, while in others all four cells differed from one another, the tetrad having, for example, one cell with each of the four combinations of the shape and chloroplast characters. The existence of such 'tetratype' tetrads showed that segregation of character-differences could occur at either division of meiosis and was in agreement with the hypothesis that crossing-over occurred at the four-strand stage, although convincing evidence of this would require linked factors.

Soon afterwards tetrad analysis was achieved in bryophytes and in basidiomycete and zygomycete fungi, but it was not until Lindegren (1933)

found linked characters in the ascomycete fungus *Neurospora crassa* that direct evidence was obtained by tetrad analysis that crossing-over occurs at the four-strand stage and involves only two of the four strands at any one place. *Neurospora crassa* is a haploid fungus existing in two mating-types. Meiosis occurs immediately after fusion of the nuclei of opposite mating-type. Wilcox (1928) had shown that in the closely allied species *N. sitophila* the four haploid nuclei resulting from meiosis are linearly arranged within an elongated tube-like cell, the ascus, such that the two second-division spindles do not overlap (see Fig. 8.7). Subsequently mitosis takes place in each

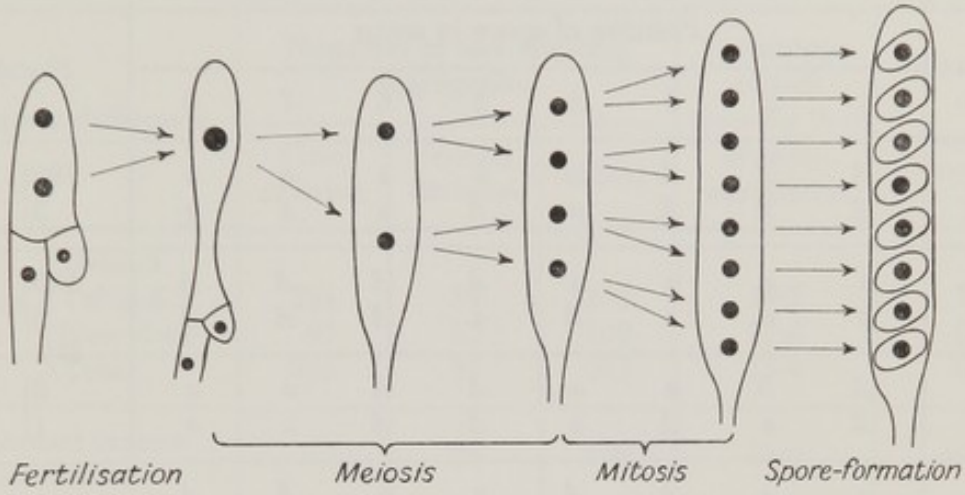


FIGURE 8.7 Stages in the development of the ascospores of *Neurospora sitophila.*

nucleus, and again the spindles do not overlap. Spore walls are then laid down round each nucleus, and the mature ascus, which is about 150 μ long and 15 μ wide, contains a linear sequence of 8 black elliptical spores, each measuring about 25 μ in length and 15 μ in width. She dissected a number of asci using a fine needle and isolated the spores in sequence. When the resulting cultures were tested for mating-type four were always of one mating-type and four of the other. Moreover, spores 1 and 2 numbering from the top of the ascus were always alike, and so were 3 and 4, and so on. In other words, the spores were in identical pairs, as would be expected if the third nuclear division in the young ascus was mitotic (cf. Pl. 4(*b*)–(*d*)).

Dodge (1929) made similar ascus dissections and found evidence that the mating-type factors sometimes segregate at the first division of meiosis and sometimes at the second division. This was confirmed by Lindegren (1932) who dissected numerous asci of *N. crassa* and tested the spores for mating-type. His results are shown in Table 8.2. Eleven out of 273 asci showed one or more pairs of non-identical spores, numbering from the end of the ascus, in contrast to what was expected if the cytology was similar to that of *N. sitophila.* However in every instance exchanging the position of two adjacent spores would suffice to explain these anomalies, and he presumed that either third-division spindle overlap or spore displacement during dissection was responsible. The 273 asci can then be classified into six classes numbered I to VI in the table. Classes I and II correspond to

segregation of *A* from *a* at the first division of meiosis, if the nuclear behaviour is like *N. sitophila*. The numbers of asci in these two classes were not significantly different (observed frequencies 105 and 129, expected 117, deviations 12, $\chi^2 = 2{\cdot}5$, which with 1 degree of freedom, is associated with a probability of just over 0·1 of obtaining a deviation as large or larger by chance). Classes III to VI correspond to segregation of *A* from *a* at the second division

TABLE 8.2 Lindegren's data (1932) for the segregation of the mating-type factors in asci of *Neurospora crassa*. The letters *A* and *a* denote the two mating-types.

Class	Position of spore in ascus								Number	
	1	2	3	4	5	6	7	8		
I	*A*	*A*	*A*	*A*	*a*	*a*	*a*	*a*	102	105
	A	*A*	*A*	*a*	*A*	*a*	*a*	*a*	3	
II	*a*	*a*	*a*	*a*	*A*	*A*	*A*	*A*	123	129
	a	*a*	*a*	*A*	*a*	*A*	*A*	*A*	6	
III	*A*	*A*	*a*	*a*	*A*	*A*	*a*	*a*	8	9
	A	*a*	*A*	*a*	*A*	*A*	*a*	*a*	1	
IV	*a*	*a*	*A*	*A*	*a*	*a*	*A*	*A*		5
V	*A*	*A*	*a*	*a*	*a*	*a*	*A*	*A*	10	11
	A	*a*	*A*	*a*	*a*	*a*	*A*	*A*	1	
VI	*a*	*a*	*A*	*A*	*A*	*A*	*a*	*a*		14
							Total			273

of meiosis, and again the numbers in the classes do not differ significantly (observed frequencies 9, 5, 11 and 14, expected 9·75, deviations 0·75, 4·75, 1·25 and 4·25, $\chi^2 = 4{\cdot}5$, which for three degrees of freedom implies a probability of between 0·5 and 0·1 of by chance getting a deviation as large or larger than that observed). However the frequencies of classes I and II clearly differ significantly from those of the other four.

Lindegren (1933) reported further data from ascus dissection in *N. crassa*. He crossed a strain of the fungus with pale aerial filaments and conidia (asexual spores) with one lacking conidia, which he described as fluffy from the appearance of the aerial growth. The normal fungus has orange-coloured conidia. Each of the characters (pale and fluffy) appeared to differ from normal by a single gene, for the spores from each ascus produced four pale and four orange cultures, and they could also be classified for the presence or absence of conidia and again there were four progeny per ascus in each class. Lindegren dissected 109 asci showing segregation for these characters. The numbers of asci with first- and second-division segregation

which he obtained are shown in Table 8.3. The frequency of second-division segregation for mating-type in this set of data (11·0%) is slightly lower than that found previously (14·3%), but the difference is not significant. (Contingency $\chi^2 = \frac{382[(234 \times 12) - (39 \times 97)]^2}{273 \times 109 \times 331 \times 51} = 0{\cdot}74$, which, with one degree of freedom means that P is just under 0·5, or in other words, a

TABLE 8.3 Lindegren's data (1933) for first- and second-division segregation frequencies for various character-differences in *Neurospora crassa*.

Character difference		Numbers of asci showing segregation			Percentage of asci showing 2nd division segregation	Distance of gene from centromere
		First division	Second division	Total		
Mating-type	Data of Table 8.2	234	39	273	14·3	7·1
	New data	97	12	109	11·0	5·5
	Total	331	51	382	13·4	6·7
Pale/normal orange conidial colour		73	36	109	33·0	16·5
Fluffy/normal growth-form		42	67	109	61·4	30·7

difference as large or larger than that found may be expected to occur by chance nearly every other time on the average.) On the other hand the second-division segregation frequencies of the pale and fluffy characters (33·0% and 61·4% respectively) clearly differ significantly from each other and from that for mating-type.

Lindegren's hypothesis to explain these results was that crossing-over occurred at the four-strand stage and involved only two of the four chromatids, and furthermore that the paternal and maternal centromeres of the chromosome pair segregated from one another at the first division of meiosis. Second-division segregation of a pair of alleles would then be the consequence of crossing-over between their locus and the centromere of the chromosome, while first-division segregation would mean that the factors had separated along with the centromeres, without crossing-over in between (Fig. 8.8). Owing to the variability in the position of cross-overs a given pair of alleles would sometimes separate at the first division of meiosis and sometimes at the second division. Lindegren pointed out that it followed from this that the frequency of second-division segregation for a particular character-difference was a measure of the frequency of crossing-over between the gene concerned and its centromere, and hence was a measure of their distance apart on the linkage map.

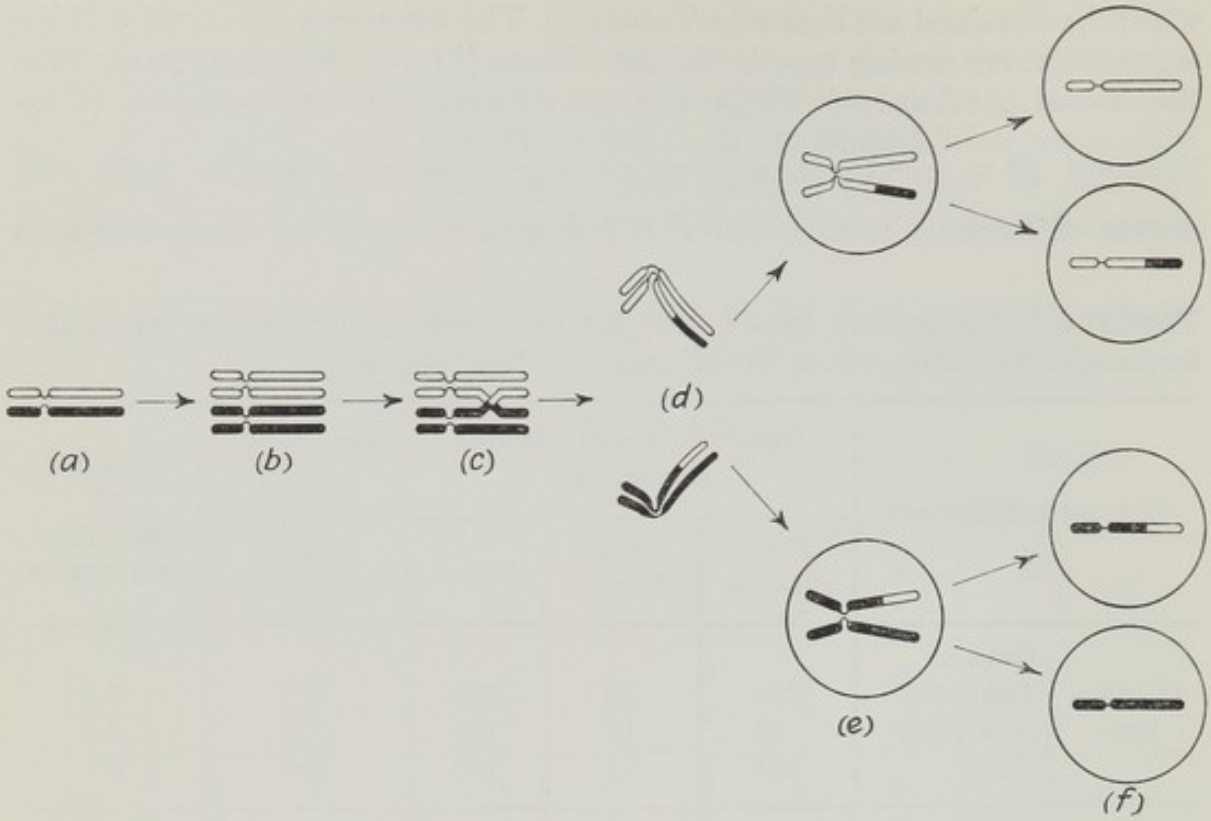


FIGURE 8.8 Diagrams of a pair of homologous chromosomes at various stages of meiosis to illustrate Lindegren's hypothesis of centromere segregation at the first division of meiosis, and crossing-over at the four-strand stage. This hypothesis was put forward to account for the characteristic frequency of second-division segregation for each gene. (*a*) Pachytene stage. (*b*) End of pachytene stage. (*c*) Diplotene stage. (*d*) Anaphase I. (*e*) Telophase I. (*f*) Telophase II.
The constrictions indicate the centromeres, and the shading the parental origin.

It will be recalled that Sturtevant (1913) had proposed that the unit of distance between two gene loci on a linkage map should be such as to give rise to 1% of cross-over gametes (see § 7.3). In a haploid organism such as *Neurospora* meiosis leads directly to the next generation, so the percentage frequency of progeny with non-parental combinations of two character-differences is the measure of the distance apart on the linkage map of the factors concerned. However, if crossing-over occurs at the four-strand stage, one exchange will lead to two cross-over and two non-cross-over progeny (compare Fig. 8.8). Hence every time a pair of allelomorphs segregate from one another at the second division of meiosis, the four products of meiosis will comprise two cross-overs and two non-cross-overs for the interval between the locus of the alleles and the centromere. Thus 1% of cross-over progeny, which is the unit on the map, will be produced by second-division segregation in 2% of meioses, or in other words frequencies of second-division segregation expressed as percentages can be converted into gene-centromere map distances simply by halving them. The centromere distances obtained in this way for the factors studied by Lindegren are given in the right-hand column of Table 8.3.

TABLE 8.4 Lindegren's data (1933) for the numbers of parental and non-parental combinations of character-differences in the 436 pairs of spores from 109 asci of *Neurospora crassa*.

Pair of Character-differences	Parental	Non-parental	Percentage Recombination
Mating-type and pale/normal (orange) conidial colour	338	98	22·5
Mating-type and fluffy/normal growth form	222	214	49·0
Pale/normal (orange) conidial colour and fluffy/normal growth-form	224	212	48·6

Lindegren's theory of first-division segregation of centromeres was supported by the recombination data from his 109 asci (see Table 8.4). The numbers of parental and non-parental associations of mating-type and fluffy growth-form (222 parental and 214 non-parental) do not differ significantly, and nor do those for the pale and fluffy characters (224 parental and 212 non-parental). On the other hand, the mating-type and pale characters showed partial linkage, with 22·5% recombination. It follows that the genes responsible for these character-differences are located 22·5 units apart on the same linkage map. This figure agrees well with the sum of their centromere distances (22·0) (see Fig. 8.9). Lindegren concluded that the genes for mating-type and pale conidia are located in opposite arms of the chromosome, and that his hypothesis of first-division segregation of centromeres, together with crossing-over at the four-strand stage and involving only two of the four strands, was correct (cf. Pl. 4(*c*)).

The hypothesis of first-division segregation of the centromeres, which is required by the chiasmatype theory, had been proposed earlier by Bridges and Anderson (1925), but their evidence was indirect and less convincing. An examination of their data and those of Anderson (1925) in Table 8.1 shows that there are progressive changes in the frequencies of the various

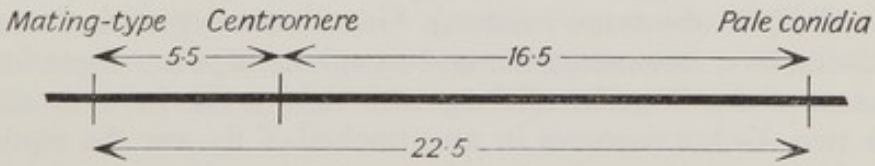


FIGURE 8.9 Linkage map of the mating-type chromosome of *Neurospora crassa*, showing the similarity between the distance apart of the mating-type and 'pale' gene loci based on second-division segregation frequencies (figures above the line) and that based on recombination frequency (figure below the line). The data are from the analysis of 109 asci by Lindegren (1933).

genotypes as one proceeds from column to column across the table, or in other words, as one proceeds along the chromosome from region to region (see Fig. 8.10). By extrapolation, it appeared that at a point beyond the gene

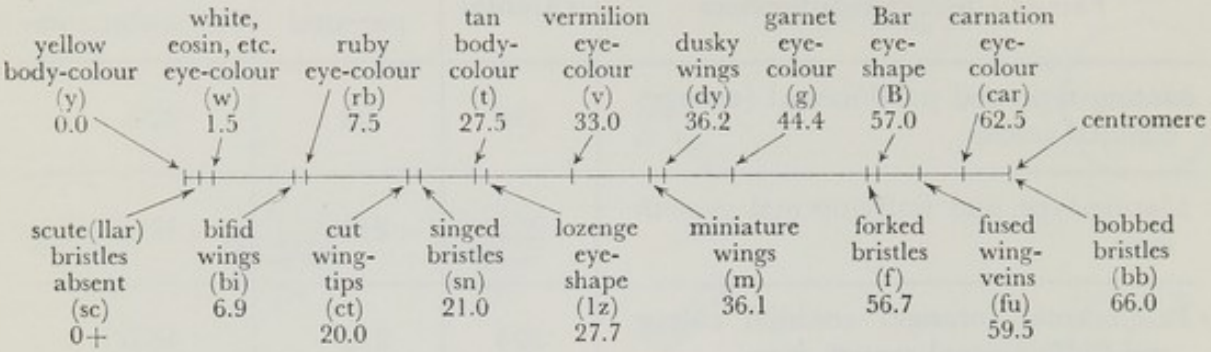


FIGURE 8.10 Linkage map of the *X*-chromosome of *Drosophila melanogaster* to show the relative positions of genes mentioned in the text. The figures show the distance from the distal end in map units. (Data chiefly from Morgan, 1926.)

for forked bristles, which in both experiments marked one end of the part of the chromosome under study, the genotypes homozygous for the *A* or the *a* allele (that is, classes (i), (ii), (vii) and (viii)) would diminish to zero. It was suggested that at this hypothetical point alleles (such as *A* and *a*) always segregated from one another at the first division of meiosis and sister genes (such as *A* and *A*) always passed to opposite poles at the second meiotic division. Since the *X*-chromosome in *Drosophila melanogaster* is rod-shaped, the centromere must lie at or near one end, and it was natural to suggest that this corresponded with the hypothetical point of first-division segregation.

With the attached-*X* flies this point of zero frequency of homozygotes would be the place where the two *X*-chromosomes joined, but this was thought to coincide with the centromere since, as already indicated, the chromosome when fixed and stained appeared V-shaped with two equal arms. Anderson's data allowed this point of junction to be mapped. From Table 8.1, out of the 188 flies studied, 22 (11·7%) were homozygous at the 'forked' locus (classes (i), (ii), (vii) and (viii) in the 'forked and garnet' column) and 48 (25·5%) were homozygous at the 'garnet' locus (classes (i), (ii), (ix) and (x) in the same column). Since homozygosis is thought to be due to crossing-over between gene and attachment-point, these frequencies are a measure of the distances of the gene loci from this point on the linkage map. The two *X*-chromosomes in an attached-*X* fly are the equivalent of two spores from opposite ends of a *Neurospora* ascus. Homozygous flies (*A*/*A* or *a*/*a*) are equivalent to second-division segregation asci, but only half the occurrences of second-division segregation are detected. This is evident from examination of Table 8.2: there are 25 asci (classes V and VI) in which spores 1 and 8 are identical, compared with a total of 39 in which second-division segregation has occurred. Since frequencies of second-division segregation must be halved for use as map distances, homozygosis frequencies, which are equivalent to the half values, can be used directly as map distances

between gene loci and the point of junction of the two *X*-chromosomes. This has been done in Fig. 8.11.

Out of the 376 *X*-chromosomes in Anderson's 188 flies, 54 (14·4%) were cross-overs between 'forked' and garnet' (classes (vii) to (x), plus twice class (iv) in Table 8.1). This frequency is close to the difference between the other two (see Fig. 8.11). Thus the recombination and homozygosis data agree,

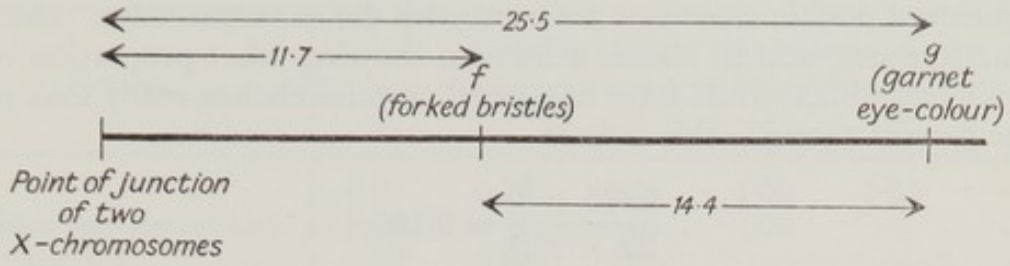


FIGURE 8.11 Linkage map of part of the *X*-chromosome of *Drosophila melanogaster* showing the similarity between the distance apart of the 'forked' and 'garnet' gene loci based on homozygosis frequencies (figures above the line) and that based on recombination frequency (figure below the line). The data are from the analysis of 188 attached-*X* females by Anderson (1925).

and suggest that the point of junction of the two *X*-chromosomes is about 12 units beyond the 'forked' locus. This corresponds well with the position of the terminal gene known (bobbed bristles) in the linkage group (see Fig. 8.10).

Support for the hypothesis of first-division segregation of centromeres could also be obtained from the observations of Carothers (1913) at spermatogenesis in the grasshopper *Brachystola magna*. As mentioned in § 6.9, she found an unequal chromosome-pair, and the inequality always segregated at the first division of meiosis. Carothers concluded that the spindle-fibres became attached at the unequal end at metaphase I (see Fig. 6.4).

The discovery that crossing-over takes place, not between paternal and maternal chromosomes, but between individual daughter chromatids, one of paternal and one of maternal origin, constituted strong evidence in support of Janssens' chiasmatype theory. However, the data which led to this discovery provided no information about crossing-over in relation to chiasmata. The next important question, therefore, in testing the truth of Janssens' theory, was to establish whether or not the frequencies and distribution of cross-overs in groups of linked genes were in agreement with the frequencies and distributions of chiasmata in the corresponding chromosomes.

§ 8.7 Genetical interference

Muller (1916) pointed out that the occurrence of one crossing-over in *Drosophila* interferes with the coincident occurrence of another in the same pair of chromosomes, and termed the phenomenon *interference*. For example, Morgan's data for 2205 flies in Table 7.3 indicate 29 cross-overs (1·3%) between the loci for yellow body-colour and white eyes, and 719 (32·6%) between the loci for white eyes and miniature wings. The expected frequency

of double cross-overs, if crossing-over occurs at random, is given by the product of these frequencies, or $\frac{29}{2205} \times \frac{719}{2205} = 0{\cdot}43\%$. The observed frequency of double cross-overs was $\frac{1}{2205}$ or $0{\cdot}045\%$. Muller suggested that the most useful way to measure the intensity of interference was to divide the observed double cross-over frequency by the expected value (assuming no interference), and so obtain a fraction showing what proportion of the coincidences which would have happened on pure chance really took place. In the present example this ratio is

$$\frac{2205 \times 1}{29 \times 719} = 0{\cdot}106.$$

He called this quantity the *coincidence*, pointing out that a low value such as this means much interference. He showed that in the *X*-chromosome of *Drosophila* the intensity of interference is very great over short intervals, but falls off with distance, such that for regions 40 units or more apart in the linkage group the coincidence has risen to near 1. Morgan (1919) called the phenomenon of interference the *fifth law of heredity*. Genetical interference has since been found to occur in most organisms that have been investigated. Thus, in *Zea*, from Stadler's data in Fig. 7.4(*a*), the frequency of double cross-overs expected if there is no interference is

$$pq = \frac{1065}{45{,}832} \times \frac{9141}{45{,}832},$$

or 212·4 out of the 45,832 progeny. The observed frequency was only 32. Hence, the coincidence is $\frac{32}{212{\cdot}4} = 0{\cdot}150$.

Stevens (1936) pointed out that for non-adjacent regions of a chromosome, the coincidence had frequently been wrongly estimated. He showed that the best estimate, whether the regions under study were adjacent or not, was given by the standard expression

$$\frac{n \times n_{pq}}{n_p \times n_q}$$

where n is the total number of individuals, and n_p, n_q, and n_{pq} are, respectively, the numbers of individuals showing a recombination in segment p, in segment q, and simultaneously in segments p and q, but with the important corollary that these numbers must be irrespective of the occurrence of recombination elsewhere. Stevens calculated coincidence values from published recombination data for *Drosophila melanogaster*. Some of his results are given Table 8.5. It is evident that within the one arm of the *X*-chromosome there is strong interference over short intervals, declining to no interference (coincidence 1) at about 40 units separation, as Muller had found. On the other hand, between the two arms of chromosome II he found there was no interference.

TABLE 8.5 Coincidence values obtained by Stevens (1936) from published recombination data for two linkage groups in *Drosophila melanogaster*. The six intervals between gene loci in linkage-group I averaged 9.4 units in length. The interval spanning the centromere in linkage-group II was 3 units long and the other six averaged 17·3 units. The coincidence values in the lower part of the table, and for three and four intervening segments in the upper part, do not differ significantly from unity.

Region	Number of Intervening Segments					
	0	1	2	3	4	5
Within one arm of linkage-group I (*X*-chromosome)	0·03	0·13	0·55	1·00	1·01	—
	0·06	0·37	0·87	1·06	—	—
	0·12	0·64	0·82	—	—	—
	0·24	0·71	—	—	—	—
	0·19	—	—	—	—	—
Across the centromere of linkage-group II	—	1·01	1·05	0·97	1·11	0·89
	—	—	1·00	0·97	0·97	—
	—	—	—	0·98	—	—

§ 8.8 Chromatid interference

With the discovery that crossing-over is between daughter chromatids and not parent chromosomes, ideas about interference had to be revised. It could no longer be implicitly assumed that interference meant that the position of one exchange between homologous chromosomes affected the position of a neighbouring exchange. Haldane (1931) pointed out that if adjacent cross-overs were usually between different pairs of chromatids, forming a four-strand double cross-over as in Fig. 8.12, a moderate degree of genetical interference was compatible with a random distribution of the points of exchange. This is possible because four-strand double cross-overs lead only to single cross-over progeny, since no chromatid is broken more than once. Mather (1933*a*) gave the name of *chromatid interference* to the phenomenon of non-random distribution between the chromatids involved in successive cross-overs. There are four possible relationships between the strands concerned in two cross-overs, and they are illustrated in Fig. 8.12. There are two kinds of three-strand double cross-overs, depending on which parent contributes the unbroken strand. If crossing-over is at random as regards the strands involved, the four types will occur with equal frequency, or in other words, the ratio of two-strand : three-strand : four-strand double cross-overs will be as 1 : 2 : 1.

Emerson and Beadle (1933) were the first to obtain appreciable data concerning the strand-relationship of adjacent cross-overs. They used attached-*X* *Drosophila melanogaster* heterozygous for several sex-linked characters, and like Anderson (1925) determined the frequencies of the various genotypes in the progeny of a cross. With three or more pairs of

alleles segregating simultaneously, it is possible to distinguish between two classes of double cross-overs: (*A*) two-strand and one kind of three-strand, and (*B*) four-strand and the other kind of three-strand. Anderson (1925) found 3 progeny of type *A* and 4 of type *B*. Emerson and Beadle, from their

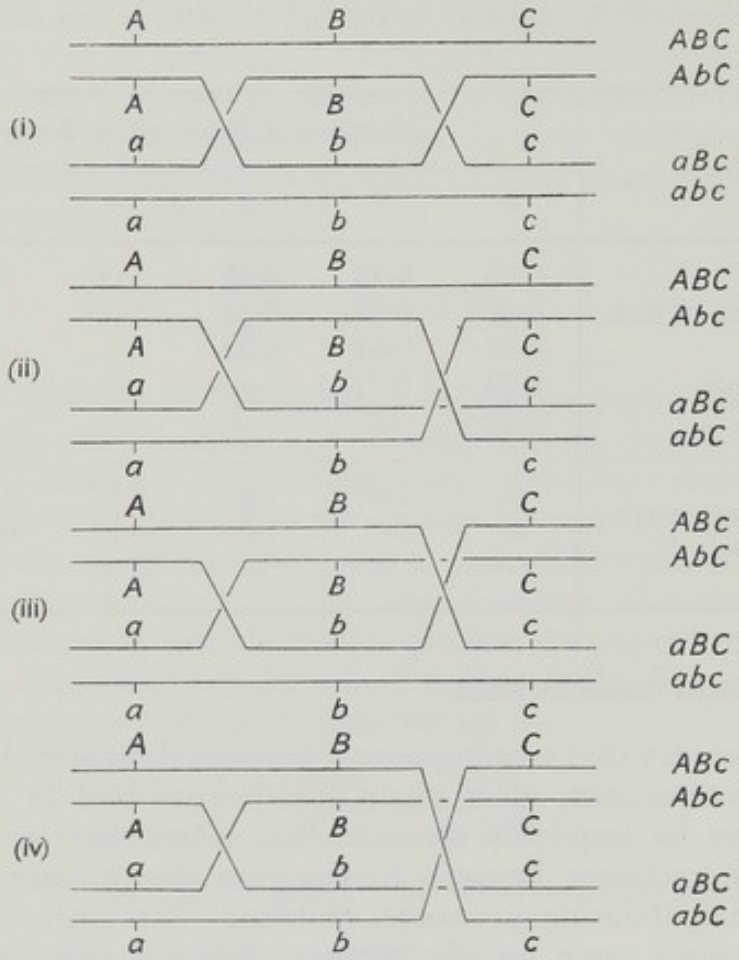


FIGURE 8.12 The four kinds of double cross-overs and the corresponding tetrad genotypes from the cross *ABC* × *abc*. (i) Two-strand, (ii) and (iii) three-strand, and (iv) four-strand relationship (cf. Pl. 4(*d*)).

own data together with unpublished data of Sturtevant, brought these totals to 73 *A*, 62 *B*. The frequencies of the two types do not differ significantly, and the inference was that, as far as the evidence went, there was no chromatid interference in this chromosome.

Tetrad analysis with three or more linked factors provides full information on the strand-relationship of cross-overs. This is evident from the fact that the four tetrad genotypes in Fig. 8.12 are all different. The first data of this kind were obtained by Lindegren with *Neurospora crassa*. Extensive results have been obtained subsequently with this organism by a number of authors, and Bole-Gowda, Perkins and Strickland (1962) quote totals of 423 two-strand, 759 three-strand and 329 four-strand double cross-overs as the pooled results of a number of recent studies. This represents a significant

excess of two-strand double cross-overs over four-strand. Data obtained by Knapp and Möller (1955) with *Sphaerocarpos donnellii*, Strickland (1958) with *Aspergillus nidulans*, and Ebersold and Levine (1959) with *Chlamydomonas reinhardi* indicate no chromatid interference, but in some instances there was an excess of two-strand double cross-overs, which, in view of the *Neurospora* results, might be found to be significant with more extensive data.

The strands involved in adjacent cross-overs can sometimes be determined cytologically. Some of the most extensive information of this kind was obtained by Brown and Zohary (1955) with *Lilium formosanum*, and is described in § 8.15. It indicated no chromatid interference within a chromosome arm.

§ 8.9 Cross-over position interference

If, as would thus appear, chromatid interference is commonly absent, it follows that genetical interference between cross-overs must often be wholly attributed to the position of one point of exchange interfering with the position of a neighbouring exchange, as indeed was originally supposed. To this phenomenon, Mather (1933*a*) gave the name 'chiasma interference'. Carter and Robertson (1952) suggested that a more explicit title would be 'chiasma position interference'. Both these names are open to the objection that they use a cytological term, chiasma, to describe a genetical phenomenon. In other words, by using these terms one assumes that the chiasmatype theory is true. A more appropriate title would be 'cross-over position interference' or simply *position interference*.

§ 8.10 Chiasma position interference

If chiasmata correspond to cross-overs, as the chiasmatype theory demands, and if cross-overs show position interference, then it is to be expected that chiasmata will also show position interference. Haldane (1931) demonstrated this, using data of Maeda (1930) for chiasma frequencies in the pollen-mother-cells of *Vicia faba*. The 12 somatic chromosomes of this plant comprise five pairs of about equal size and with sub-terminal centromeres, and one pair about twice as long and with the centromere near the middle. Maeda recorded the numbers of chiasmata at metaphase I in 1100 of the five smaller chromosome-pairs (which were not individually distinguishable) and in 1057 of the large one. The mean chiasma frequencies were 3·5 in the short chromosomes and 8·1 in the long one. Haldane drew attention to the distribution of chiasma frequencies about these mean values. In the short chromosomes, a majority had three or four chiasmata and the range was from 1 to 6, while in the long one there was a great concentration at 7 to 9 chiasmata and the total range was from 3 to 13. He pointed out that if the distribution of chiasmata was at random along the chromosomes, the frequencies of chromosomes with different numbers of chiasmata would fit a Poisson distribution, in which the variance is equal to the mean. Analysis of Maeda's data showed that for both the short chromosomes and the long

one there was much less variation in chiasma frequency than would be expected on the Poisson distribution. Thus, for the short chromosomes the variance was only about one quarter of the mean and for the long one about one third. These differences from random chiasma distribution were highly significant and indicated that the occurrence of one chiasma was greatly reducing the likelihood of another in its vicinity. Haldane obtained similar results from the analysis of less extensive data for several other flowering plants.

Mather (1933*a*) made the converse analysis. He took published recombination data for linkage groups II and III of *Drosophila melanogaster*, assumed there was no chromatid interference and that chiasmata corresponded to points of crossing-over between two chromatids, and determined the chiasma distribution that would result. He found that the *Drosophila* genetical data would lead to a chiasma distribution in which the variance was $\frac{1}{6}$ to $\frac{1}{4}$ of the mean, in good agreement with the cytological data from Angiosperms.

§ 8.11 Chiasma frequencies and cross-over frequencies

The pattern of distribution of chiasmata in chromosomes thus appears to be in agreement with the hypothesis that chiasmata correspond to the points of crossing-over. However, if the chiasmatype theory is true, one would also expect to find correspondence between chiasma frequencies and cross-over frequencies in individual chromosomes. Morgan, Sturtevant, Muller and Bridges (1915) reported that mutant genes in *Drosophila melanogaster* fell into four linkage groups. Within groups there were various intensities of partial linkage, while between groups parental and non-parental combinations were statistically equal in frequency. These findings agreed with the observation that the haploid chromosome number was 4. Morgan (1919) called this equality between numbers of linkage groups and of chromosomes the *sixth law of heredity*. Although at the time established only for *Drosophila*, later work has confirmed the truth of this law for many organisms including for example *Zea mays* with 10 linkage groups and chromosomes, and *Neurospora crassa* with seven of each. Morgan *et al.* (1915) also found that the number of mutants in each *Drosophila* linkage group was roughly proportional to the physical size of the corresponding chromosome.

Darlington (1934*b*) showed that there was agreement between chiasma and cross-over frequencies for each chromosome of *Zea mays*. He obtained a mean total chiasma frequency of 27·1 for the ten chromosome-pairs in the pollen-mother-cells. Distributing these chiasmata amongst the chromosomes in proportion to their lengths, using more recent estimates of the latter from Rhoades (1950), has given the figures in the third column of Table 8.6. However, Darlington found evidence that the shorter chromosomes had relatively more chiasmata in relation to their length, as indicated in the caption to the table. He compared the estimated chiasma frequencies with the cross-over frequencies derived from published linkage data. More extensive data from Rhoades (1950) are now available and are given in the fourth column of the table. With crossing-over occurring between only two of the

four chromatids, one exchange will produce 50% of cross-over gametes and 50% with the parental gene combinations. Hence 50 units on the linkage map is equivalent to an average of one exchange per meiosis in that interval of the chromosome. The figures in column 4 have therefore been obtained by taking the total length of each linkage map and dividing it by 50. In every instance the frequencies of crossing-over are slightly less than the

TABLE 8.6 Comparison of chiasma and cross-over frequencies in *Zea mays*. The data in columns 2 and 4 are from Rhoades (1950), and the total chiasma frequency is from Darlington (1934*b*), who found evidence that the short chromosomes have slightly more chiasmata in relation to their length. His estimates of the chiasma frequencies of chromosomes 1, 2, 9 and 10 would approximate to 3·8, 3·2, 2·2 and 2·0, respectively.

Chromosome Number	Length in μ at Mid-Pachytene	Estimated Mean Chiasma Frequency if Proportional to Length	Mean Cross-over Frequency of Mapped Portion
1	82	4·0	3·1
2	67	3·3	2·6
3	62	3·0	2·4
4	59	2·9	2·2
5	60	2·9	1·4
6	49	2·4	1·3
7	47	2·3	1·9
8	47	2·3	0·6
9	43	2·1	1·4
10	37	1·8	1·1
Totals	553	27·0	18·0

chiasma frequencies (on average two thirds of them), but this is to be expected since it is unlikely that genes have been mapped at the extreme ends of the chromosomes. This agreement with expectation gives further support to the view that chiasmata correspond to the points of crossing-over, and hence supports the chiasmatype hypothesis.

The probability that a newly-obtained mutant will show linkage to a previously known mutant will depend on the chromosome number and the cross-over frequency for that organism. Carter (1955) has shown how total cross-over frequencies per mieosis can be estimated from such linkage data, and has applied the method to data for *Mus musculus* (Mouse). Of 33 mutants, 9 were due to genes situated within 25 units of a previously discovered mutant, from which the total cross-over frequency per meiosis was estimated to be approximately 34. This estimate did not differ significantly from the observed total chiasma frequency for the 20 chromosome pairs, which was approximately 38.

The evidence presented so far in this chapter has established that crossing-over occurs between two of the four chromatids; that these chromatids

appear to be chosen more or less at random at each cross-over; that paternal and maternal centromeres segregate from one another at the first division of meiosis; and that chiasmata and points of crossing-over show similar frequencies of occurrence and corresponding intensities of interference. All these findings are in agreement with Janssens' chiasmatype theory, but nevertheless do not constitute convincing evidence of the truth of the theory.

§ 8.12 Belling's modification of Janssens' theory

Janssens' theory, although favoured almost at once by geneticists, received no support from other cytologists for nearly 20 years. They criticised it on the grounds that they could find no evidence for the breakage and rejoining of

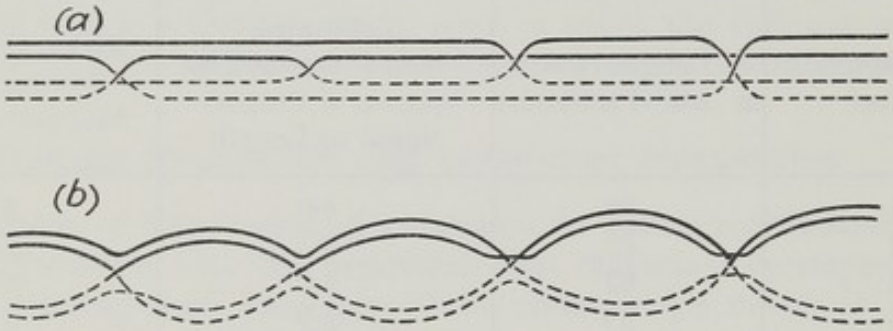


FIGURE 8.13 Diagrams to illustrate Belling's modification of Janssens' hypothesis of exchange between homologous chromosomes. Chromatid segments derived from one parent are shown with a broken line and those derived from the other parent by an unbroken line. (*a*) Pachytene stage. (*b*) Diplotene stage.

threads at the early diplotene stage, such as he had claimed to have seen. However, Belling (1928*a*), after studying the meiotic chromosomes in various flowering plants, proposed a modification of Janssens' theory which removed this criticism. He suggested that the exchange between chromatids took place at the preceding pachytene stage, while the homologous chromosomes were closely associated. Exchange at this stage could not be directly observed, but the chiasmata seen subsequently would be the consequence of these exchanges, if one accepted, as Janssens had done, that throughout the length of each chromosome of the bivalent association, the pairing at the diplotene stage was always between daughter-chromatids derived from the same parent chromosome (Fig. 8.13). Belling (1929) gave two primary reasons for believing that the diplotene pairing was of this kind.

§ 8.13 Unequal chromosome pairs

Firstly, whenever homologous chromosomes differed in appearance at one end, as had been observed by Carothers (1913), Wenrich (1916) and others in various Orthoptera, and by Belling himself in *Aloe purpurascens*, the two short chromatid-ends were always associated with one another at the

diplotene stage and similarly with the two long ones (Fig. 8.14(*a*)). This was true whether or not the centromere was close to the end where the difference occurred. Darlington (1932, pp. 274–277) took this argument a stage further. He pointed out that since on the chiasmatype hypothesis sister-chromatids are always associated at the diplotene stage, the loop or pair of arms containing the centromeres will have two paternal chromatids associated at one centromere region and two maternal at the other. Since these centromere regions move to opposite poles at anaphase I, paternal and maternal centromeres will always show first-division segregation, just as Bridges & Anderson (1925) had postulated on genetic evidence. If there is no chiasma between the centromere and a region of the chromosome where the homologues differ, then this difference will segregate at the first division of meiosis, as found by Carothers (1913) with *Brachystola magna*. On the other hand, a single chiasma between centromere and inequality would lead to second-division segregation for the region of difference.

An example of regular second-division segregation for an inequality was found by Wenrich (1916) at spermatogenesis in the grasshopper *Phrynotettix tschivavensis*. Chromosome-pair *B* was found in 11 individuals to show heterozygosity for the presence and absence of a large terminal granule. Wenrich observed a single medianly-placed chiasma in each *B* chromosome-pair. Since the two chromatid-ends with the granule were always associated with one another, and similarly with the two without the granule, and since Wenrich states that he found no indication of breaking or recombining of the parts of chromatids, he must have assumed that one paternal and one maternal centromere went to each pole at the first division. On the other hand, Darlington suggested there was first-division segregation of centromeres and that crossing-over had occurred at the position of the chiasma.

In two individuals of *Phrynotettix*, Wenrich found a similar inequality in chromosome-pair *C*, but here it was variable in behaviour (Fig. 8.14(*b*) and (*c*)). Out of 928 cells which he examined at metaphase I, 472 showed first-division segregation for the inequality and the remaining 456 (49·2%) showed second-division segregation. As with chromosome-pair *B*, the metaphase configuration was cross-shaped, implying a single medianly-placed chiasma. Wenrich suggested that the centromeres were situated at the end of the chromosome showing the inequality when this segregated at the first-division of meiosis, and that they moved to the other end with second-division segregation. Darlington put forward the suggestion that the centromere was fixed in position, that it was medianly placed, that the chiasma might occur in either arm, and that crossing-over preceded its appearance. If crossing-over occurred in the opposite arm to the inequality, first-division segregation of the latter would occur, and if in the same arm there would be second-division segregation. However, the centromere appeared to be subterminal and not median in position.

Nur (1961) has offered a solution to this dilemma. He discovered in another short-horned grasshopper, *Calliptamus palaestinensis*, that an inequality in a chromosome-pair, although often appearing terminal, was really interstitially placed (Fig. 8.14(*d*) and (*e*)). The centromere was sub-terminal

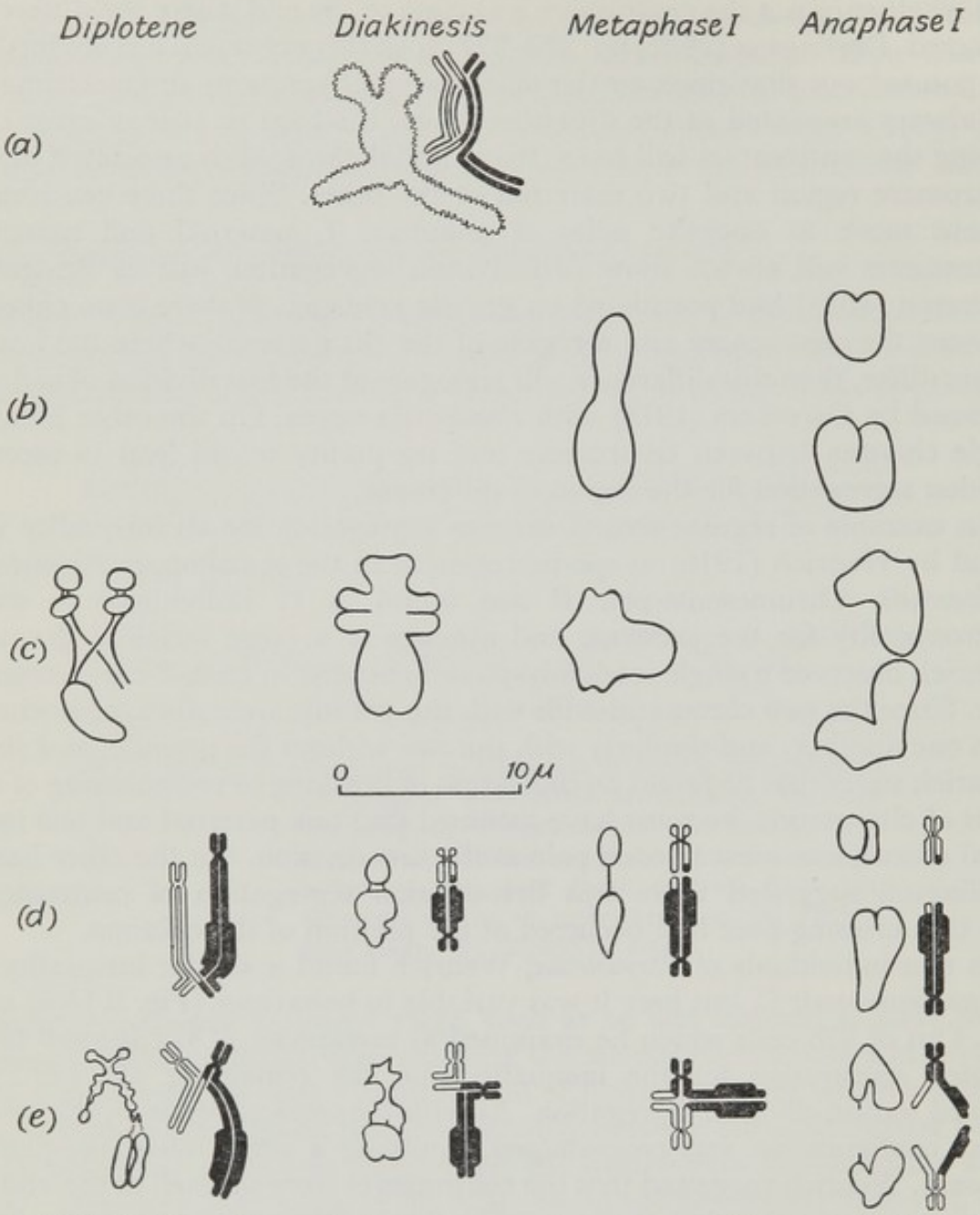


FIGURE 8.14 Unequal chromosome-pairs at stages of the first division of meiosis.
(*a*) *Aloe purpurascens* (from Belling, 1928*c*).
(*b*) and (*c*) *Phrynotettix tschivavensis* chromosome-pair *C* (from Wenrich, 1916).
(*d*) and (*e*) *Calliptamus palaestinensis* (from Nur, 1961).
(*b*) and (*d*) First-division segregation for the inequality.
(*c*) and (*e*) Second-division segregation for the inequality.
The diagrams to the right of the drawings in (*a*), (*d*) and (*e*) show the interpretation of the observed structures according to the chiasmatype hypothesis, with the black and white segments indicating the parental origin.

in position and at meiosis in the male a single chiasma occurred regularly and appeared always to be in the long arm, just as in chromosome-pair *C* of *Phrynotettix* studied by Wenrich. Out of 410 cells in *Calliptamus* at the diplotene or diakinetic stage, Nur found that the chiasma appeared to be proximal to the inequality in 379 (92·4%) and distal to it in the remaining 31. Examination of 69 cells at anaphase I showed 63 (91·3%) with second-division segregation for the inequality and 6 with first-division segregation, in agreement with the observations at the earlier stage. It is presumed that the inequality in chromosome-pair *C* of *Phrynotettix*, studied by Wenrich (1916), was also interstitially placed although appearing terminal, and that chiasmata occurred proximal and distal to it equally often.

Several authors have made quantitative studies with unequal chromosome-pairs showing variability of behaviour like Wenrich's pair *C*. Their results are given in the upper part of Table 8.7. In each study the interval between centromere and region of inequality was short and never showed more than a single chiasma. With the exception of Haga's data for *Paris verticillata*, there was no significant difference (contingency χ^2 value, for one degree of freedom, less than 4—see last column of Table 8.7) between the chiasma frequency observed at diakinesis or metaphase I, and the second-division segregation frequency observed at anaphase I or II. A remarkable feature of meiosis in *P. verticillata* is the regular occurrence of chiasmata close to the centromere in either or both arms of each chromosome. It is therefore exceptionally difficult material in which to record the frequency of cells with a chiasma between the centromere and the inequality. In Haga's data, 667 cells had a chiasma in the unequal arm and 333 were thought to show chiasmata only in the other arm, but owing to the close proximity of the centromere to these chiasmata it is probable that some of them were in fact in the unequal arm. Hence the excess of second-division segregations (82·25%) over chiasmata (66·7%) is expected.

The data in the lower part of Table 8.7 refer to individuals heterozygous for the presence and absence of an exchange of part of an arm between two different (that is, non-homologous) chromosomes. Such individuals will have two chromosome-pairs each with an unequal arm. If the two chromosomes and their exchanged portions are similar in appearance, it may not be possible to recognise them individually, as was found by Kayano (1960*a*) with *Disporum sessile* and by Noda (1961) with *Scilla scilloides*. In any event, in such interchange heterozygotes the recognition of the unequal pairs at diakinesis or metaphase I requires skill, since the two pairs will commonly be held together by chiasmata to form a quadrivalent association. As with the simple inequalities discussed in the last paragraph, the intervals between centromere and inequality were short and except in chromosome I of *Acrida lata* (see table) never showed more than one chiasma. Without exception, no significant differences were found between the chiasma frequencies and the corresponding second-division segregation frequencies.

On the chiasmatype hypothesis, paternal and maternal centromeres segregate at the first division of meiosis, and the segregation of a chromosomal inequality at the second division can only be accounted for by

TABLE 8.7 Frequencies of cells with and without a chiasma between the centromere and an inequality in a chromosome-pair, and frequencies of first- and second-division segregation for the inequality. In the right-hand column the contingency χ^2 values are given from a comparison of the chiasma and segregation frequencies. χ^2 values of less than about 4 indicate no significant difference between chiasma frequencies and second-division segregation frequencies.

Type of Structural Hetero-zygote	Organism and Reference	Chromo-some Pair	Diakinesis or Metaphase I				Anaphase I or II				χ^2 $n = 1$
			Without Chiasma	With Chiasma	Total	Percentage with Chiasma	1st Division Segregation	2nd Division Segregation	Total	Percentage 2nd Division Segregation	
Simple inequality	*Mesocricetus auratus* Koller, 1938	*X* & *Y*	206	47	253	18·6	87	24	111	21·6	0·46
	Paris verticillata Haga, 1944	*D*	333	667	1000	66·7	355	1645	2000	82·25	91·3
	Lilium formosanum Brown & Zohary, 1955	*A**	183	425	608	70·0	69	172	241	71·4	0·18
			114	120	234	51·3	67	81	148	54·7	0·43
		*I**	21	150	171	87·7	15	123	138	89·1	0·15
			72	273	345	79·1	43	149	192	77·6	0·17
	Allium fistulosum Zen, 1961	*S*	151	618	769	80·4	61	237	298	79·5	0·087

Non-homologous interchange	*Lilium maximowiczii* Noda, 1960	J_1	490	110	600	18·3	981	219	1200	18·25	0·0019
		J_2	600	0	600	0	1200	0	1200	0	0
	Disporum sessile Kayano, 1960*a*	*A*	1549	651	(1100 / 1100)	Mean 29·6	1987	853	(1420 / 1420)	Mean 30·0	0·12
		B									
	Acrida lata Kayano & Nakamura, 1960	*I*	0	50†	50	97·0†	2	28	30	93·3	0·60
		II	9	41	50	82·0	3	27	30	90·0	0.94
	Allium fistulosum Zen, 1961	*S*	59	208	267	77·9	17	50	67	74·6	0·33
		F	138	129	267	48·3	29	25	54	46·3	0·073
	Scilla scilloides Noda, 1961	*a*	1128	2820	(1974 / 1974)	Mean 71·4	278	678	(478 / 478)	Mean 70·9	0·097
		b									

*The two sets of data for chromosome A of *Lilium formosanum* refer to different individuals heterozygous for the same chromosomal deficiency, while those for chromosome I refer to the same individual in different years.

†In chromosome I of *Acrida lata* there were 47 cells with single and 3 with double chiasmata. Assuming there was no chromatid interference, only half the double chiasmata would lead to second-division segregation.

postulating crossing-over between the centromere and the region of difference. Hence the finding that second-division segregation frequencies are statistically equal to chiasma frequencies is equivalent to finding that crossing-over and chiasma frequencies are equal, and so provides strong support for the chiasmatype theory. Furthermore, the invariable occurrence of pairing at late prophase and metaphase I between the two long segments, and similarly with the two short segments making up the inequality, even when a single chiasma has occurred between the inequality and the centromere, shows that these chiasmata are not merely points where the four threads have changed partners, but that breakage and crossing-over have occurred.

Inequalities in diplotene chromosome-pairs in oocytes of *Triturus cristatus* (Crested Newt) observed by Callan and Lloyd (1956) have provided direct evidence in support of the chiasmatype theory. The chromosomes at this stage are greatly elongated and have a remarkable 'lampbrush' structure (see § 17.6 and Pl. 6(b)), in which the two chromatids of which they are composed extend individually into lateral loops. The four chromatids in a chromosome-pair usually showed loops of similar appearance at corresponding positions, but in each meiotic cell in certain individuals a particular loop was absent from two of them. It was evident that these individuals were heterozygous for a difference at this point in the chromosome. The significant observation was that the two chromatids which possessed this particular loop were paired, and similarly with the two without the loop. A number of such differences between homologues were observed, and in each instance it was evident that sister-chromatids were paired at the diplotene stage, as expected on the chiasmatype theory.

§ 8.14 Polyploids

Belling's second main reason for believing that the diplotene pairing was between sister-chromatids was to account for configurations of three homologous chromosomes which he had observed at meiosis in triploid horticultural varieties of *Hyacinthus orientalis*. Belling (1929) and Darlington (1929*b*) discovered with triploid *Hyacinthus* and Newton and Darlington (1929) with triploid *Tulipa* that although for each chromosome all three homologues came together at the zygotene stage of meiosis, only two showed close pairing in any one region of the chromosome (cf. § 6.4). However, at intervals there were changes of partner. Thus a typical pachytene trivalent association is like that shown diagrammatically in Fig. 8.15(*a*) with two changes of pairing partner. Belling thought that the chiasmata seen at the diplotene stage in a trivalent chromosomal association could not have arisen by an exchange of pairing partner, but required crossing-over to explain their origin.

Here also the argument was taken further by Darlington (1930) who demonstrated with polyploid *Hyacinthus* that a chiasma was the consequence of previous crossing-over. This demonstration was based on the observation that a chromosome may show two chiasmata *A* and *B* (see Fig. 8.15(*b*)–(*d*)) with one homologue, and a third chiasma *C* with another homologue at a

point between *A* and *B*. To account for such a configuration it is necessary to assume that at the earlier pachytene stage there were changes of pairing partner as in Fig. 8.15(*a*). On this assumption, chiasmata *A* and *B* must involve the same four chromatids, and therefore chiasma *C* must involve

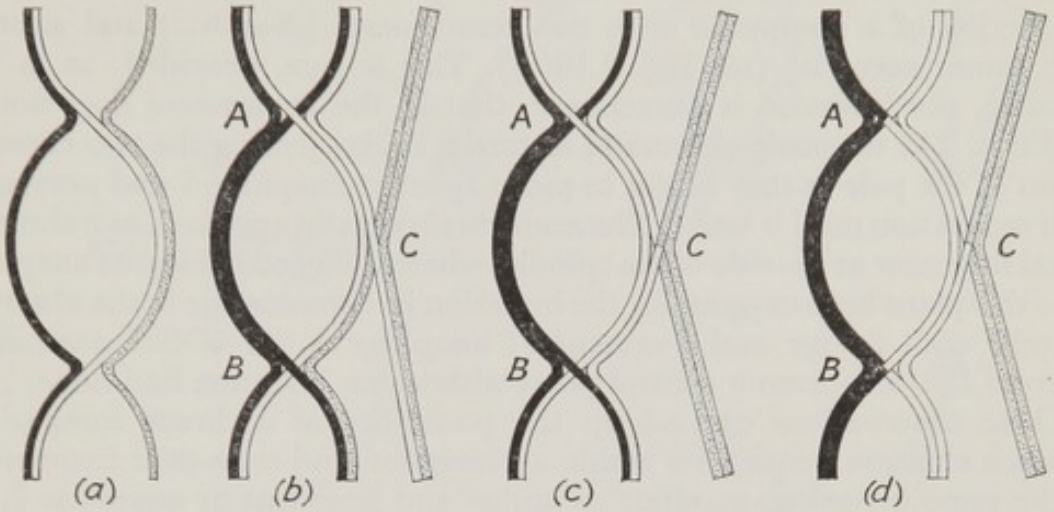


FIGURE 8.15 Diagrams of a trivalent chromosomal association to illustrate Darlington's demonstration that crossing-over must precede chiasma-formation.
(*a*) Pachytene stage.
(*b–d*) Alternative explanations of diplotene configuration with three chiasmata.
(*b*) With all chiasmata due to changes of pairing partner without breakage (classical hypothesis).
(*c*) With chiasmata *A* and *B* classical, but *C* due to breakage and crossing-over (chiasmatype hypothesis).
(*d*) With all chiasmata due to crossing-over (chiasmatype hypothesis).

breakage and crossing-over (see Fig. 8.15(*b*) and (*c*)). Critical evidence is not available for chiasmata *A* and *B* but there is no reason to suppose they differ from *C*. Such intermediate chiasmata with a third homologue are of frequent occurrence at meiosis in polyploid organisms. Since the pachytene association in all polyploids appears to be strictly confined to pairs over any given region of a chromosome, it follows that all such intermediate chiasmata with a third homologue must be the consequence of previous crossing-over.

§ 8.15 Chromosomal inversions

A third important source of critical information about chiasmata in relation to crossing-over followed from the discovery by McClintock (1931) that if a segment of a chromosome has become relatively inverted, individuals heterozygous for such an inversion show reversed pairing at the pachytene stage. Following *X*-irradiation of pollen, she obtained a plant of *Zea mays* heterozygous for a long inversion in chromosome 2. It occupied parts of both arms and included about two-thirds of the total length of the chromosome. Characteristic loops were formed at the pachytene stage in the

pollen-mother-cells, with the two homologous chromosomes passing along the loop in opposite directions, such that homologous parts were in association (see Fig. 8.16(i) and (ii)). Later (McClintock, 1933) in untreated *Zea* she found a plant heterozygous for an inversion within the short arm of chromosome 8 and she showed that crossing-over within the inversion led to the production of a chromatid with two centromeres (dicentric) and another with none (acentric) (see Fig. 8.16(v)). This is true, provided, as in this instance, the inversion is paracentric, that is, the centromere does not lie within it. The dicentric chromatid formed a bridge joining the two chromosomes of the pair as they began to move apart at anaphase I and prevented their separation until it broke. The acentric chromatid appeared as a chromosomal fragment at the side of the spindle, where it lagged behind at anaphase I. In this plant heterozygous for the inversion in chromosome 8, she observed 25 cells with bridge and fragment at anaphase I out of 281 examined, whereas 123 cells from a control plant without an inversion had none.

These observations opened up the possibility of a direct comparison between chiasma frequencies within an inversion and cross-over frequencies in the same inversion, manifest as bridge and fragment at anaphase I. In making such a comparison it is necessary to take into account the consequences of double crossing-over. In her *Zea* plant heterozygous for the chromosome 8 inversion, McClintock (1933) found one instance of the chromosome-pair at anaphase I joined by two dicentric bridges and with two acentric fragments alongside. She explained this as due to a four-strand double cross-over within the inversion. Smith (1935) found a wild plant of *Trillium erectum* which was heterozygous for a short inversion near the middle of the long arm of chromosome *D*. In about 9·5% of the pollen-mother-cells at metaphase I or anaphase I, a dicentric bridge and an acentric fragment about 6 μ long were present. In a further 2·5% of the mother-cells the fragment was present without the bridge, but then a dicentric loop could be seen at metaphase I, which gave a bridge at anaphase II. He explained the first-division loop by postulating one cross-over within the inversion and a second cross-over between the inversion and the centromere, such that one chromatid was involved in both cross-overs, that is, there was a three-strand double cross-over (see Fig. 8.16(vi)–(viii)). The proximal cross-over, that is, the one between inversion and centromere, then has the effect of converting the first-division bridge into a loop.

Smith's observations form the basis of a cytological method, referred to earlier in this chapter, of testing for chromatid interference. With no chromatid interference, a cross-over in a paracentric inversion and another proximal to it will give rise to equal frequencies of bridges at anaphase I (two- or four-strand relationships) and anaphase II (three-strand relationships between the two cross-overs). The marked excess of first-division bridges (9·5%) over loops (2·5%) in Smith's data for *Trillium erectum* may be due to the occurrence of cross-overs within the inversion without a proximal cross-over, rather than to an excess of two- and four-strand relationships over three-strand. On the other hand, Brown and Zohary (1955) following *X*-irradiation of pollen, obtained plants of *Lilium formosanum* heterozygous

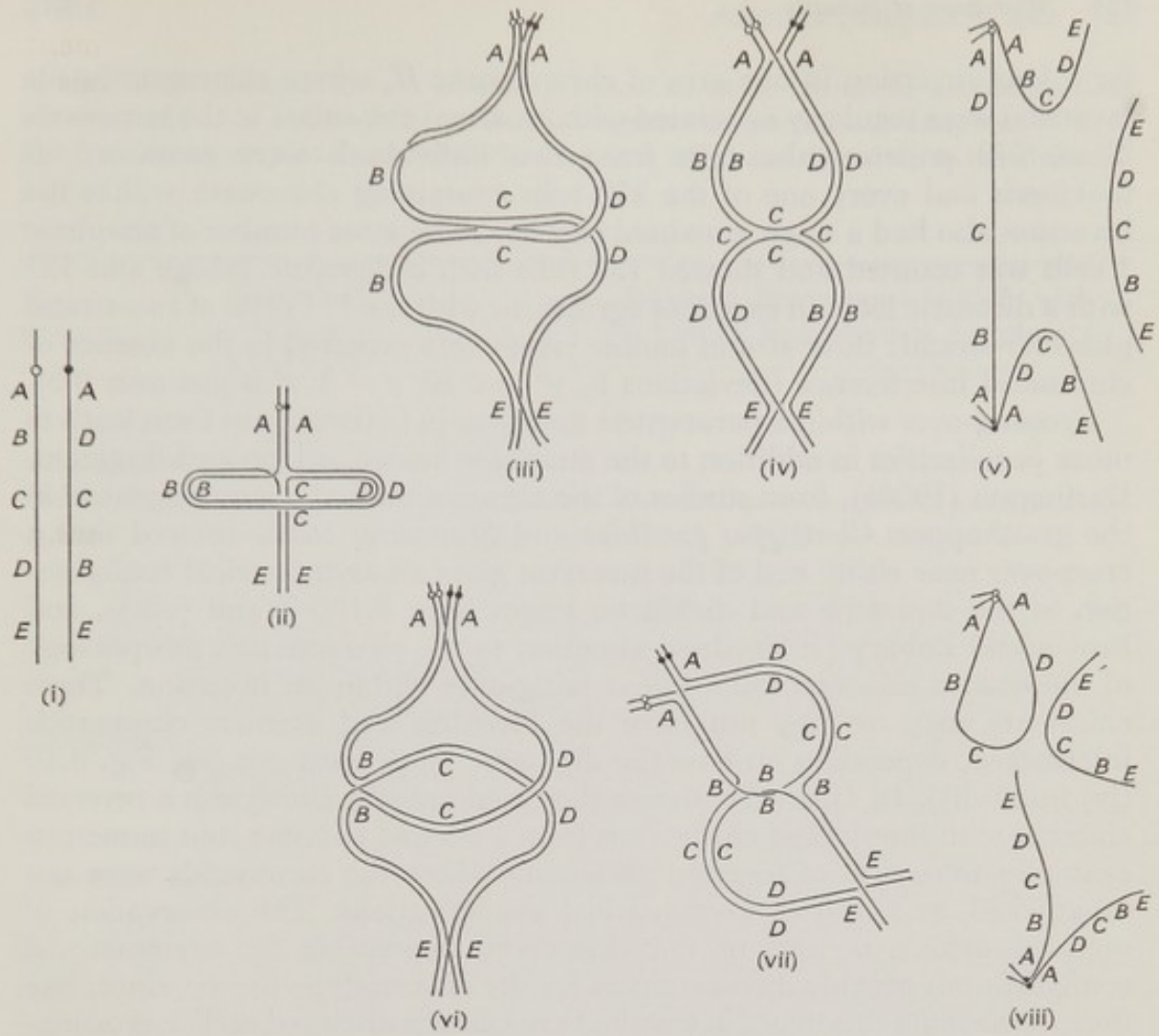


FIGURE 8.16 Diagrams to show stages of meiosis in a chromosome-pair heterozygous for an inversion in the middle of one arm. *A B C D E* represent different regions of the chromosome arm. The normal sequence is alphabetical, but in the inversion the regions *B*, *C* and *D* are reversed to give the sequence *A D C B E*. A hollow circle represents a centromere derived from one parent and a solid circle from the other.

(i) Leptotene stage.

(ii) Pachytene stage with pairing in a loop such that homologous regions are associated.

(iii) and (iv) Diplotene stage with chiasmata at *A*, *C* and *E*. The chiasma at *C* is in the inversion and is of characteristic appearance, best seen in (iv). The cross-over at *C* involves the same two strands (or the other two) as that at *A*.

(v) Anaphase I, with dicentric bridge and acentric fragment, such as would result from the configurations shown in (iii) and (iv).

(vi) and (vii) As (iii) and (iv), but the chiasma in the inversion is at *B* instead of *C*, giving an asymmetrical configuration, and the cross-overs at *A* and *B* involve three strands altogether instead of two or four.

(viii) Anaphase I, with dicentric loop and acentric fragment, such as would result from the configurations shown in (vi) and (vii).

The dicentric and acentric chromatids are shown interlocked at the reversed chiasma in (vii), but not in (iv). Whether or not they will be interlocked is thought to be a matter of chance.

for a long inversion in one arm of chromosome *H*, where chiasmata in the inversion were regularly associated with proximal chiasmata in the same arm. Thus, 278 pollen-mother-cells from two individuals were examined at diakinesis and every one of the 252 cells containing chiasmata within the inversion also had a single proximal chiasma. The same number of anaphase I cells was counted and showed 131 cells with a dicentric bridge and 121 with a dicentric loop, in excellent agreement with the 1 : 1 ratio of two-strand plus four-strand: three-strand double cross-overs expected in the absence of chromatid interference (deviations 5, $\chi^2 = 0{\cdot}40$, $n = 1$, P is just over 0·5).

Crossing-over within a paracentric inversion in heterozygous form leads to other peculiarities in addition to the anaphase bridge or loop and fragment. Darlington (1936*b*), from studies of the chromosomes at spermatogenesis in the grasshoppers *Chorthippus parallelus* and *Stauroderus bicolor* showed that a cross-over near either end of the inversion gives an asymmetrical configuration at the diplotene and diakinetic stages (Fig. 8.16(vi) and (viii)), and Brown and Zohary (1955) drew attention to the characteristic morphology of the *reversed chiasmata* following crossing-over within an inversion. These chiasmata may or may not have the dicentric and acentric chromatids interlocked, depending on how the diplotene loops open out (see Fig. 8.16 (iv) and (vii)). In *Lilium formosanum* they could rarely distinguish a reversed chiasma with interlocked chromatids from a normal chiasma, but numerous examples were seen of reversed chiasmata where the chromatids were not interlocked, and also of asymmetrical configurations. The observation of reversed chiasmata and of the chiasmata responsible for asymmetrical configurations provide direct support for the chiasmatype theory, since, like the intermediate chiasmata in trivalent associations discussed earlier, crossing-over must have preceded their formation.

The direct comparison between chiasma frequencies in a paracentric inversion, observed at diakinesis, and bridge (or loop) and fragment frequencies, observed at anaphase I, has been made by Brown and Zohary (1955) with *Lilium formosanum*. Their data show an excellent agreement between chiasma and cross-over frequencies. In one plant of *L. formosanum* heterozygous for the chromosome *H* inversion 145 cells were examined at diakinesis. There were 17 without chiasmata in the inversion, 67 with one chiasma and 61 with two chiasmata. On the chiasmatype hypothesis and assuming there was no chromatid interference, two chiasmata within the inversion would be the consequence of two-strand, three-strand and four-strand double cross-overs in the ratio 1:2:1. The two-strand double cross-overs would give no bridges and fragments, as though without crossing-over in the inversion, since the two cross-overs would in effect cancel out. The three-strand double cross-overs would give one bridge and fragment, and the four-strand double cross-overs would give two bridges and fragments. Hence, the expected frequencies of cells with 0, 1 and 2 fragments at anaphase I can be calculated (see Table 8.8(*a*)). It was found that there was agreement between observed and expected numbers of fragments at anaphase I. A second plant also showed agreement: see Table 8.8(*b*). Thus Brown and Zohary's data provide strong support for the chiasmatype theory.

TABLE 8.8 Comparison of chiasma frequencies at diakinesis in an inversion in chromosome *H* of *Lilium formosanum* and fragment frequencies at anaphase *I*, from the data of Brown and Zohary (1955). The data in (*a*) and (*b*) relate to two different plants.

(*a*)

Diakinesis			Expected Percentages of Cells with 0, 1 and 2 Fragments at Anaphase *I*		
No. of Chiasmata	Observed Nos. of Cells (Total 145)	Percentage Cells	0	1	2
0	17	11·7	11·7	—	—
1	67	46·2	—	46·2	—
2	61	42·1	10·5	21·0	10·5
Anaphase I: Expected percentages			22·2	67·2	10·5
Anaphase I: Expected numbers of cells (total 121)			26·9	81·3	12·8
Anaphase I: Observed numbers of cells (total 121)			21	80	20
$\chi^2 = 5{\cdot}5$, $n = 2$, $P = 0{\cdot}1$–$0{\cdot}05$					

(*b*)

Diakinesis			Expected Percentages of Cells with 0, 1 and 2 Fragments at Anaphase I		
No. of Chiasmata	Observed Nos. of Cells (Total 133)	Percentage Cells	0	1	2
0	9	6·8	6·8	—	—
1	55	41·3	—	41·3	—
2	69	51·9	13·0	25·9	13·0
Anaphase I: Expected percentages			19·8	67·2	13·0
Anaphase I: Expected numbers of cells (total 197)			38·8	132·6	25·6
Anaphase I: Observed numbers of cells (total 197)			45	123	29
$\chi^2 = 2{\cdot}1$, $n = 2$, $P = 0{\cdot}1$–$0{\cdot}5$					

§ 8.16 Additional cytological evidence for the chiasmatype theory

Critical cytological evidence for the occurrence of chiasmata as a consequence of previous crossing-over is available from several additional sources, but unlike the three main lines of evidence discussed above, these others have only rarely been observed, or have not been subjected to sufficient quantitative analysis. Darlington (1931*c*) reported figure-of-eight

shaped chromosomal configurations at meiosis in *Oenothera* species, in which the chiasma at the centre of the '8' could only be interpreted as due to previous crossing-over between a pair of chromosomes which were heterozygous at both ends for interchanges of segments with non-homologous chromosomes. Mather (1933*b*) described an instance where two of the 12 chromosome-pairs at meiosis in a pollen-mother-cell of *Lilium regale* were interlocked such that a medianly placed chiasma could only have arisen as a consequence of previous crossing-over, and in *L. henryi* and *L. japonicum* he several times observed (Mather 1935) a chiasma between a chromosome fragment and a normal chromosome, again implying previous crossing-over. Darlington (1935) observed coiling of chromosomes round one another on each side of a chiasma at the diplotene stage of meiosis in a species of *Fritillaria*, and deduced that the chiasma must have been the consequence of crossing-over, but this argument is valid only on the assumption that the coiling of the chromosomes round each other had arisen before they divided into chromatids.

Kayano (1960*b*) obtained an individual of *Disporum sessile* heterozygous for structural differences at both ends of the long arm of chromosome *A*: the presence or absence of an interchange with chromosome *B* at one end, and the presence or absence of a terminal knob (satellite) at the other. Study of this chromosome in 1100 pollen-mother-cells at metaphase I of meiosis showed 537 (48·8%) without chiasmata, 381 with 1 chiasma, 163 with 2 chiasmata, and 19 with 3 chiasmata. Assuming the chiasmatype hypothesis is true, and that there was no chromatid interference, the expected frequencies

TABLE 8.9 Comparison of chiasma frequencies at metaphase I in the long arm of chromosome *A* of *Disporum sessile* with the frequencies of different combinations at anaphase II of structural features at each end of the arm, from the data of Kayano (1960*b*).

Metaphase I			Expected Percentages of Cells with Different Combinations at Anaphase II of the 4 Copies of Chromosome *A*		
No. of Chiasmata	Observed Nos. of Cells (Total 1100)	Percentage Cells	All 4 Parental	2 Parental 2 Recombined	All 4 Recombined
0	537	48·8	48·8	—	—
1	381	34·6	—	34·6	—
2	163	14·8	3·7	7·4	3·7
3	19	1·8	0·2	1·4	0·2
Anaphase II	Expected percentages		52·7	43·4	3·9
	Expected numbers of cells (total 80)		42·2	34·7	3·1
	Observed numbers of cells (total 80)		43	34	3
			$\chi^2 = 0{\cdot}034$, $n = 2$, $P = 0{\cdot}99$–$0{\cdot}98$		

can be calculated of anaphase II cells in which the 4 chromosome *A*'s resembled those of the parent, or in which 2 of them showed recombination of the characters by which their ends differed and 2 did not, or in which all 4 showed recombination (see Table 8.9). The observed numbers of these 3 classes of anaphase II cells were in close agreement—indeed alarmingly close agreement—with the expected values (see table). The expected frequency of recombinant chromosome *A*'s is $\frac{1}{2}(100 - 48{\cdot}8)\% = 25{\cdot}6\%$, and the observed number in 1671 chromosome *A*'s examined at anaphase II was 419 or 25·1%, again in very close agreement ($\chi^2 = 0{\cdot}23, n = 1, P = 0{\cdot}9$–$0{\cdot}5$).

§ 8.17 The hypothesis of crossing-over by breakage of classical chiasmata

The observations discussed above provide overwhelming evidence for a connection between chiasmata and crossing-over, but before drawing the conclusion that crossing-over is the cause of a chiasma, it is necessary to consider another hypothesis which relates chiasmata to crossing-over. Wenrich (1916), although not favouring breakage of chromosomes, thought that at the point where two chromatids cross each other in a chiasma, which he regarded merely as a point where chromatids change partners, a weakness of the strands might cause them to break and then recombine to give cross-over chromatids. This hypothesis of crossing-over through breakage of classical chiasmata has been revived on many occasions. It is open to the objection advanced by Muller (1916) against Janssens' theory, namely, that the precision needed in crossing-over is difficult to account for if the breakage and rejoining occur when the chromosomes are relatively short and thick, as at the diplotene stage. Indeed this difficulty is still more serious if the breakage occurs even later, as Wenrich's theory would suppose, since at diakinesis the chromosomes reach their maximum state of contraction. However, the theory appeared to find support from a reduction in the number of chiasmata between the diplotene stage and metaphase I in many organisms.

§ 8.18 Terminalisation of chiasmata

This support was removed when Darlington (1931*a*), following a study of meiosis in *Primula sinensis*, showed that the reduction in numbers could be accounted for by postulating that the chiasmata moved to the ends of the chromosomes and replaced one another there, without breakage (see Fig. 8.17). This process of *terminalisation*, as he called it, is found in some organisms, particularly those with small chromosomes, but not in others. In *P. sinensis* Darlington found from 2 to 5 interstitial chiasmata in each diplotene chromosome-pair, but at diakinesis and metaphase I all the chiasmata had terminalised and hence there were only two per bivalent association (or only one if no chiasmata had occurred in one arm). On the chiasmatype theory, terminalisation implies that the position of the chiasmata will no longer coincide with the points of crossing-over (Fig. 8.18).

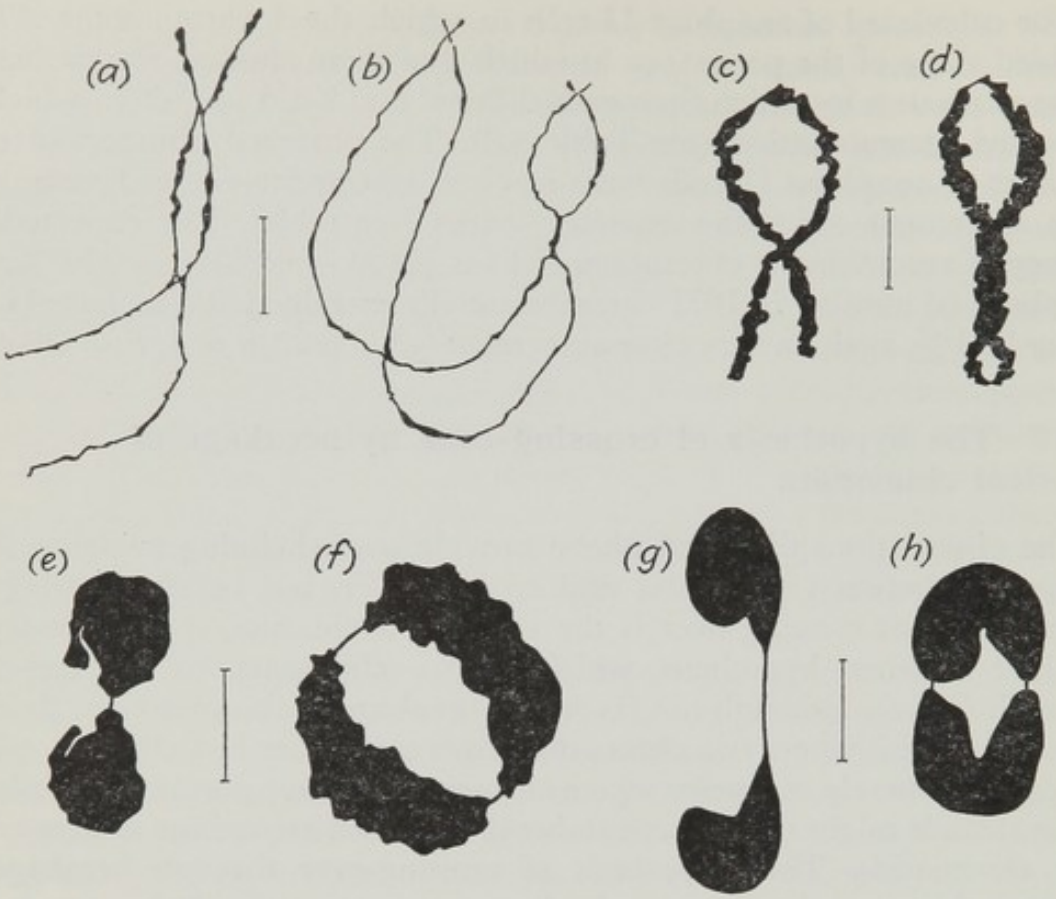


FIGURE 8.17 Drawings of chromosomes of *Primula sinensis* to show terminalisation of chiasmata (from Darlington, 1931*a*). The vertical lines represent 1 μ.
(*a*) and (*b*) Early diplotene stage.
(*c*) and (*d*) Late diplotene stage.
(*e*) and (*f*) Diakinesis.
(*g*) and (*h*) Metaphase I.

The acceptance of the notion of chiasma movement led to the final discrediting of the idea that the chromosomes associate end to end instead of side by side in early prophase I of meiosis. This idea had been maintained, at least for some organisms, for nearly half a century. The end-to-end pairing had never been observed but was inferred from the end-to-end association seen at diakinesis and metaphase I in, for example, *Oenothera*.* Darlington's theory of terminalisation of chiasmata explained these late prophase I terminal associations as of secondary origin from an earlier lateral association (Darlington 1929*a*, 1931*c*). The recognition of the terminal chiasma led Darlington to two further hypotheses: firstly (1929*b*) that it is chiasmata, either interstitial or terminal in position, that are responsible for maintaining the chromosomes in pairs between the end of the pachytene stage and the end of metaphase I; and secondly (1931*b*) that meiosis can be looked upon as a precocious mitosis, with the force which keeps the daughter-chromatids together in pairs during the prophase of

* The multiple associations of chromosomes seen at meiosis in many species of *Oenothera* are due to heterozygosity for interchanges of chromosome arms between non-homologous chromosomes (cf. § 9.2).

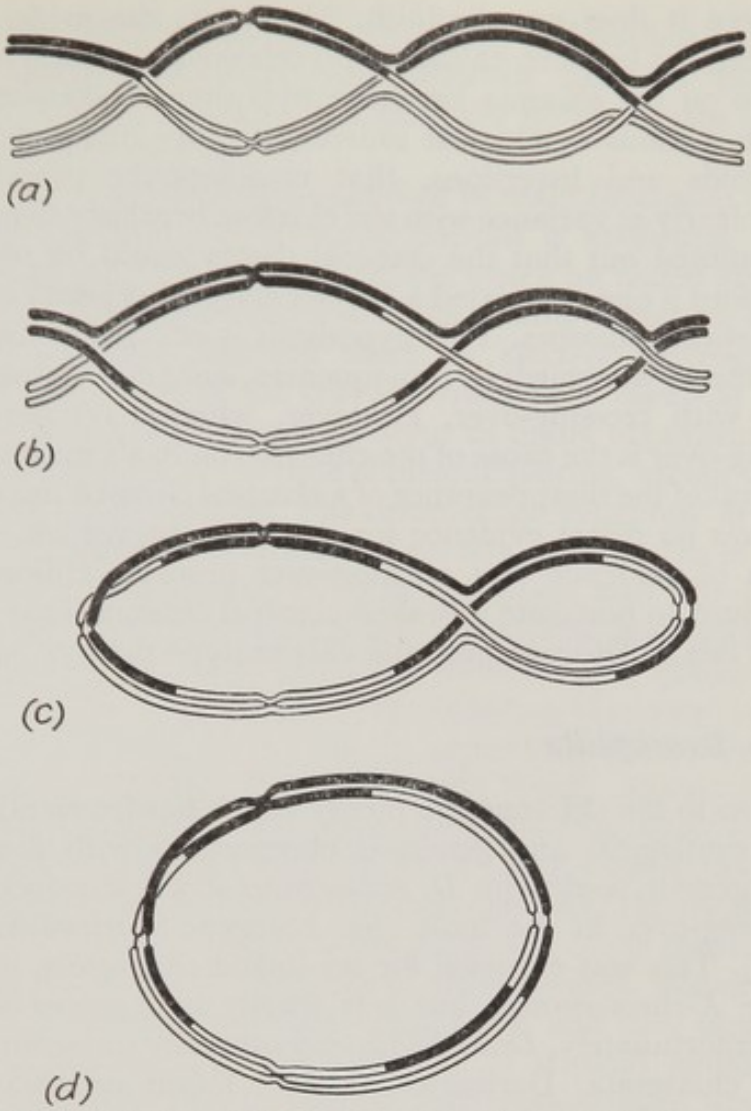


FIGURE 8.18 Diagram to show terminalisation of chiasmata. The constrictions indicate the centromeres, and the shading the parental origin.
(*a*)–(*d*) Successive stages from early diplotene to diakinesis, showing how three interstitial chiasmata become two terminal chiasmata. The points where crossing-over has taken place remain unchanged.

mitosis also responsible for the zygotene and pachytene pairing of whole undivided chromosomes and for their diplotene separation again when the chromosomes divide into chromatids. This precocity hypothesis of meiosis now appears improbable (see § 11.5).

§ 8.19 Objections to the chiasma breakage hypothesis

Darlington (1931*a*) pointed out that crossing-over takes place both in organisms such as *Primula sinensis*, where the number of chiasmata diminishes between the diplotene stage and metaphase I, and in organisms such as

Zea mays where it does not diminish. Moreover, the evidence presented earlier of agreement between chiasma and cross-over frequencies in *Zea mays* is inexplicable on the chiasma breakage hypothesis. Likewise, the critical evidence from various cytological sources, notably unequal chromosome-pairs, polyploids and inversions, that crossing-over precedes chiasma-formation, is clearly at variance with the chiasma breakage theory. However, Sax (1930) pointed out that the classical theory could be reconciled with such observations if one postulated a broken classical chiasma alongside each observed (classical) chiasma. This hypothesis is effectively the same as the chiasmatype theory as regards its consequences, since every observed chiasma is associated with crossing-over. However, whereas on the chiasmatype theory crossing-over is the cause of the chiasma, on Sax's suggestion crossing-over is the result of the disappearance of a classical chiasma near the observed one. There was no direct evidence for the latter suggestion, and with the accumulation of evidence that crossing-over precedes chiasma-formation, it became absurd to postulate a broken classical chiasma near every critical chiasma, and Sax (1936) accepted the chiasmatype theory.

§ 8.20 Male *Drosophila*

An objection to the chiasmatype theory which has frequently been raised is based on cytological and genetical observations with male *Drosophila*. Early in the genetic work with *D. melanogaster* it was discovered that there was no crossing-over in the male (see Morgan, Sturtevant, Muller and Bridges 1915). This was expected for sex-linked characters, since the male has only one *X*-chromosome, but was wholly unexpected for autosomal characters. Unfortunately, *Drosophila* is exceptionally unfavourable material for study of chiasmata. Darlington (1934*a*) found no chiasmata in the autosomes of male *D. pseudo-obscura*, the homologous chromosomes merely lying parallel to one another at diakinesis and metaphase I. In *Drosophila*, unlike most organisms, homologous chromosomes show pairing in the somatic cells as well as at meiosis, and the pairing of the autosomes at meiosis in the male was similar to this somatic association. On the other hand, Darlington found chiasmata between the *X*- and *Y*-chromosomes in the region near the centromeres where pairing takes place. In order to reconcile these observations with first-division segregation of the *X*- and *Y*-chromosomes, which is known to occur in *Drosophila*, Darlington had to postulate the regular occurrence of two chiasmata in close proximity and both in the same chromosome arm, and with a two- or four-strand relationship between their associated cross-overs. Such an elaborate explanation could hardly be said to support the chiasmatype theory. Cooper (1949) claimed to have seen chiasmata in the autosomes at meiosis in male *D. melanogaster*, and also in the somatic cells. Such chiasmata could not be associated with crossing-over, since none normally occurs in the male. Cooper concluded that these were classical chiasmata, without chromosome breakage and crossing-over. However, Slizynski (1964) has also examined the chromosomes of *D. melanogaster* males at meiosis and has found evidence that what appear to be

chiasmata in the autosomes are merely places of superficial contact. Similar chiasma-like associations were also observed between non-homologous chromosomes. It thus appears that there are no chiasmata in male *Drosophila*, not even classical ones.

Electron microscopy has confirmed that in male *Drosophila* the association between homologous chromosomes at meiosis is abnormal. Moses (1956, 1958) discovered in a crustacean structures which he called *synaptinemal complexes* because they were associated with the pairing (synapsis) of homologous chromosomes. He first observed them on electron micrographs of thin sections of spermatocytes of the crayfish *Procambarus clarkii* at prophase I of meiosis, but they are now known to occur in many organisms and seem to be of general occurrence at the time of meiotic pairing, except when crossing-over and chiasmata are lacking. The complex, or 'synapton' as it might more conveniently be called, is composed of protein and consists of two electron-dense bands (lateral elements) lying in one plane and 120–150 mμ apart, with a third band (the central element) of variable appearance depending on the species, situated midway between the other two (Moses & Coleman, 1964) (see Plate 4(*a*)). Fine threads (the transverse filaments) cross the spaces between the dark bands. The synapton apparently lies between the paired chromosomes and extends throughout their length. Moens (1968) studied its development in *Lilium longiflorum*. He found that each lateral element arises separately in early meiotic prophase as an *axial core* to the chromosome, and that it has transverse filaments down one side. He suggested that the central element arises through the inter-digitating of the transverse filaments of the axial cores of the two homologous chromosomes. Meyer (1961) found no synaptons at meiosis in male *D. melanogaster*, and they were also lacking (Meyer, 1964) in unpaired chromosomes of triploid females, and in a mutant in which chromosome pairing and crossing-over were suppressed. Moreover, in several other Diptera the presence of synaptons was associated with the occurrence of chiasmata, and lack of synaptons with their absence.

§ 8.21 Conclusion

The data presented in this chapter have provided a substantial body of evidence that chiasmata are invariably the consequence of previous crossing-over between two of the four chromatids, and hence that Janssens' chiasma-type theory, in the modified form proposed by Belling, is true.

9. *The classical theory of the gene*

§ 9.1 Multiple alleles

A discovery of great importance for the theory of the gene was made by Cuénot (1904). He found that the number of alternatives for a particular character which showed Mendelian inheritance was not limited to two. From the breeding of different strains of albino mice (*Mus musculus*), he obtained individuals with a black coat-colour, and others with a yellow coat. On crossing each of these with the normal grey he found that the black coat-colour was inherited as a Mendelian recessive and the yellow as a Mendelian dominant (with the complication that the homozygous yellow mice were apparently inviable). Furthermore, from crosses involving both the genes for black and for yellow fur, he found that these behaved as alleles towards one another, with yellow dominant to black. In other words, the genes for yellow, grey and black fur formed a series of alleles. There are six possible genotypes with respect to 3 alleles (the 3 homozygotes and the 3 heterozygotes), since a normal diploid individual necessarily cannot contain more than 2 alleles. Subsequently, the coat-colours grey with a light belly, and black with a light belly were found to belong to the same series of alleles. Normal mice are grey on the belly as well as on the back. Light belly-colour was found to be dominant to dark and grey back-colour dominant to black, so in order of descending dominance, the alleles are: Yellow; Grey with Light belly; Grey with grey belly, and black with Light belly (heterozygote Grey with Light belly); black.

Many other examples of multiple alleles were soon found. One of the first to be established in plants was through the work of Emerson (1911) with *Zea mays*. He had self-pollinated, and also inter-crossed, a number of strains of maize differing in the colour of the reproductive organs. He found that when he crossed pure-breeding strains having different colours in the axis (*cob*) of the fertilised female inflorescence, and in the ovary tissue (*pericarp*) surrounding the seed, only two classes appeared in F_2. Since the pericarp, like the cob, is parental tissue, all the grains on a maize ear will be alike as regards the pericarp colour, although there may be variation on the ear in the colour of the underlying tissues of the seed. The numbers of individuals in the two classes approximated to a 3:1 ratio, with the more frequent class having the same appearance as the F_1 generation. The deduction was clear: each pair of strains differed by one gene. Moreover, since different combinations were tested and all gave 3:1 ratios in F_2, it was evident that, in respect of the colour of these structures, the genes by which the strains differed were allelic. For example, he found that an F_1 plant with

red cob and pericarp gave an F_2 family consisting of 17 plants with red and 4 plants with white (colourless) cob and pericarp. Another red F_1 plant, from a different cross, gave in F_2 45 plants with red and 12 with variegated (red and white) cob and pericarp. A third F_1 plant, with the latter characters, gave in F_2 23 plants with variegated and 6 with colourless cob and pericarp. Evidently, red, variegated, and colourless cob and pericarp are determined by a series of 3 alleles, with red dominant to both the others, and with variegated dominant to colourless.

Over a dozen alleles are now known at this gene locus, giving different shades of red pigment (anthocyanin) and different distributions of it: for example, white cob and red pericarp; red cob and colourless pericarp; white cob and half-red pericarp (red at base, colourless at top of each grain); red cob and half-red pericarp. In every instance, the deeper shades of red were found to be dominant to the lighter ones, and the presence of colour to be dominant to its absence. Thus the heterozygote of the allele giving white cob and red pericarp with the allele giving red cob and colourless pericarp had red cob and pericarp. Moreover, dominance was found to be incomplete, a red-white heterozygote being paler than the red homozygote.

One of the best-known examples of multiple alleles was discovered by Morgan (1912) in *Drosophila melanogaster*. The white-eyed mutant of this normally red-eyed fly was found in 1910. The next year a male fly with yellowish-pink eyes described as eosin-coloured, occurred in an otherwise white-eyed family. On crossing eosin-eyed with normal red-eyed flies, the eosin character was found to show sex-linkage and to behave as a simple recessive to red. On crossing an eosin-eyed female with a white-eyed male the F_1 flies were all eosin-eyed, and in F_2 1147 eosin-eyed and 344 white-eyed flies were obtained, in agreement with a 3:1 ratio (expected frequencies 1118·25 and 372·75, deviations 28·75, $\chi^2 = 2{\cdot}95$, $n = 1$, $P = 0{\cdot}1$–$0{\cdot}05$). It was evident that the white and eosin eye-colours were inherited as a pair of alleles, with eosin dominant to white.* It was significant that in both F_1 and F_2 there were no wild-type (red-eyed) flies.

This result was in striking contrast to the results obtained when non-allelic mutants were crossed. For example, Morgan (1911*b*) had crossed a pink-eyed female with a male with bright red eyes which he described as vermilion-coloured. Both these eye-colours had been found to be inherited as recessive characters to the normal dull red eyes. All the F_1 generation flies from this cross had normal red eyes, and out of 1884 F_2 individuals, 1090 had normal red eyes, 397 had vermilion eyes, 313 had pink eyes and 84 had orange (= vermilion-pink) eyes. These figures approach a 9:3:3:1 ratio (1060:353:353:118) such as would be expected with two independently-inherited pairs of character-differences, but there is a significant shortage of flies with pink or vermilion-pink eyes, presumably due to a lowered viability of such individuals.

* There was a dosage effect for the intensity of the eosin colouring. Eosin-eyed males, and females heterozygous for eosin and white, both of which would evidently have only one eosin gene per nucleus, were found to have paler eyes than homozygous eosin females with two doses of the eosin gene per nucleus.

Further alleles at the 'white eye' locus were soon found and over a dozen are now known. They are all recessive to red and dominant to white, and include the colours described as 'cherry,' 'apricot' and 'ivory'.

The examples just described illustrate the two outstanding characteristics of a series of allelic mutants. First, all the alleles affect the same character and are non-complementary, that is to say, on crossing two of them, the F_1 generation is either intermediate in appearance between the two, or like one of them. On the other hand, non-allelic mutants, although they may affect the same character, can usually complement each other: each carries the dominant normal allele of the other's mutation (assuming the mutants are recessive), and on crossing them, the F_1 progeny are unlike either but have the normal (wild-type) phenotype of the organism.

Secondly, recombination studies indicate that allelic mutants appear to be due to genes located at the same positions in the linkage group. Thus, not only do they fail to show recombination with one another when crossed, but they each give the same recombination frequencies with other mutants. Thus, Morgan *et al.* (1915) found that the white/Red and eosin/Red eye-colour differences in *Drosophila melanogaster* both gave 1% of recombination with yellow/Grey body-colour, 33% with miniature/Normal wing-size, and 44% with Bar/normal eye-shape. On the other hand, non-allelic mutants show recombination with one another when crossed, giving in F_2 some wild-type and some double-mutant individuals, as well as the parental single-mutant types. Non-allelic mutants also differ from one another in their recombination frequencies with other mutants. The logical conclusion is that allelic mutants occupy the same gene locus in one of the chromosomes, whereas non-allelic mutants occupy different loci, either in the same or a different chromosome.

One of the best-known examples of multiple alleles concerns the four human blood-groups, O, A, B and AB. These correspond to the presence or absence of two antigens, A and B, in the erythrocytes (A—A only; B—B only; AB—both; O—neither). There is a reciprocal relationship between the presence of antigens in the red cells, and of antibodies in the serum; in other words, group O serum has antibodies against both A and B antigens, group A serum has antibodies against B, group B against A, and group AB against neither. Agglutination of cells occurs when a cell suspension and a serum are mixed, if they have an antigen and an antibody in common. Bernstein (1925) showed that the four blood-groups were inherited as Mendelian characters determined by three alleles A, B and O. Of the 6 genotypes (OO, AA, AO, BB, BO, AB), AA and AO are indistinguishable in phenotype and constitute blood group A, and similarly BB and BO are indistinguishable as group B. In Britain, the mean frequencies of the four blood-groups are approximately 46·7% for O, 41·7% for A, 8·6% for B and 3·0% for AB. Weinberg (1909) extended the Hardy-Weinberg theorem (see § 2.6) to include three alleles in a population. He showed that under the same conditions as for two alleles (that is, a large population, random mating, and no selection, migration or mutation), the frequencies of the 6 possible genotypes for three alleles are given by the terms of $(p + q + r)^2$,

where p, q and r denote the frequencies of the three alleles. If p, q and r correspond to A, B and O, respectively, applying the British frequencies given above, $r^2 = 0{\cdot}467$, $p^2 + 2pr = 0{\cdot}417$, $q^2 + 2qr = 0{\cdot}086$, and $2pq = 0{\cdot}030$, from which good estimates for p, q and r are $p = 0{\cdot}257$, $q = 0{\cdot}060$ and $r = 0{\cdot}683$. Thus, 68·3% of British ABO chromosomes carry allele O, 25·7% carry allele A, and 6·0% allele B. Further alleles (subgroups of A) have since been recognised (see Race and Sanger, 1962).

§ 9.2 Mutation

The nature of the process of mutation whereby the various alleles at a gene locus are evidently derived from one another is clearly of fundamental importance to the theory of the gene. The term mutation was introduced into biological literature by De Vries (1901). He had made extensive studies of *Oenothera lamarckiana* (Evening Primrose) and found that new forms, differing appreciably in appearance from the original, apparently arose abruptly in small numbers in each generation. On the basis of these observations, he proposed a general theory of species formation by means of sudden discontinuous changes, to which he gave the name *mutation*. Study of the cytological and genetical behaviour of this and other species of *Oenothera* by many scientists during the succeeding 30 years established that these plants have an abnormal hereditary mechanism. It appears that successive exchanges of arms between non-homologous chromosomes have taken place during the course of their evolution, until all or nearly all the chromosomes have become involved. The plants are maintained heterozygous for these interchanges of chromosome segments by adaptations which prevent the survival, or in some instances even the formation, of the homozygotes. However, these adaptations occasionally break down, and this leads to the formation of small numbers of individuals homozygous, or at least partially so, for the chromosomal interchanges present in the parent. Owing to the presence of recessive genes which thereby become manifest, these homozygotes differ in appearance from the original plant, and moreover breed true for these characters. Thus it became evident that the 'mutations' in *Oenothera* described by De Vries were really the revealing of hereditary variation already present in the parent but normally hidden, rather than changes in the genes themselves. However, De Vries' idea of abrupt changes, even if unacceptable as a general hypothesis for the evolution of new species, was required to account for the origin of the character-differences which showed Mendelian inheritance. It was natural therefore that the term should have been transferred from the species to the gene.

The existence of multiple alleles implies that a gene could mutate in more than one way. This is illustrated by the behaviour of the white-eye alleles in *Drosophila melanogaster*, first studied by Morgan. As already mentioned, the original white-eyed fly arose from the normal red, and the original eosin-eyed fly from the white. But subsequently several instances were discovered of eosin eye-colour arising directly from red. Moreover, these steps were reversible, eosin occasionally mutating back to red, and also to white. An

important feature of gene mutation was that the mutated gene was evidently replicated and passed to the progeny in the mutated form, since the altered phenotype was inherited. However, the rate at which mutations occurred was so low that it was exceedingly difficult to make detailed or quantitative studies of the process.

§ 9.3 Muller's grandsonless-lineage technique

Muller (1928*a*) described two ingenious techniques which he had devised for measuring the mutation rate in *Drosophila melanogaster*. Some 10 years previously he had come to the conclusion, on theoretical grounds, that lethal mutations might be sufficiently frequent to be studied quantitatively, since they were likely to occur considerably more often than those causing a visible change in the appearance of the organism. However, the detection of lethal mutations is more difficult than the detection of non-lethal ones. A mutation will in general not be manifest in a diploid organism such as a fly until the F_3 generation has been bred from the individual in which it occurred, since most mutations are recessive to their normal alleles. However, mutations occurring in the *X*-chromosomes of *Drosophila* are more favourably placed for detection, because they will become evident a generation sooner, causing, if lethal, the death of a fraction of the F_2 males. But even so, such mutations are not easily found, since the deaths will probably occur shortly after fertilisation, and it will be necessary to try and distinguish a real shortage of males from a chance fluctuation from the normal 1 : 1 ratio of the sexes. With often fewer than 30 progeny per female, such a distinction may not be possible.

Muller's technique was to use a known recessive lethal gene in one of the *X*-chromosomes of a female in order to discover whether another recessive lethal gene was present in the other *X*-chromosome. The known lethal gene would necessarily be in the maternally-derived *X*-chromosome since the gene would have been lethal to the male parent with its single *X*-chromosome. A new lethal mutation, if present in the other *X*-chromosome, would presumably have arisen in the sperm which fertilised the egg from which the female developed. Such a mutation would cause the female to produce no sons, since, unless a crossover occurred between the loci of the two lethal factors, the single *X*-chromosome in the male progeny would necessarily carry one or other of the two lethal genes. Crossing-over could be prevented, since strains of *Drosophila* were available in which it was largely suppressed in particular chromosomes. (This crossover suppression was later shown to be due to the presence of inversions in heterozygous form occupying the greater part of the length of the chromosome. As shown in § 8.15, crossing-over within an inversion leads to the production of chromosomes with extensive duplications and deficiencies of chromosomal segments, and consequently to inviable gametes.) Thus, Muller was able to detect the occurrence of lethal mutations in the *X*-chromosomes of the reproductive cells of male *Drosophila* by the failure of these flies to produce any grandsons when their daughters carried a known lethal factor and a crossover suppressor in the other *X*-chromosome.

§ 9.4 Muller's accumulation technique

Muller also devised a method for counting lethal mutations which allowed them to accumulate over a number of generations until significant numbers were present. With the low mutation rates usually encountered, this method was likely to be preferable to the grandsonless-lineage method. A strain of flies was used in which there were two different recessive lethal (or sterility) genes, one in each chromosome of one of the pairs other than the *X*-chromosome. One of the pair also carried a cross-over suppressor: the other was the test-chromosome. In such a strain the known lethal (or sterility) genes would automatically be maintained in heterozygous condition, since the homozygotes would not survive (or would not leave any progeny). Any additional recessive lethal mutations which appeared would be similarly maintained and could be allowed to accumulate. Their existence in the test-chromosome could be revealed when desired by crossing one fly from each test lineage to another stock, and then interbreeding the progeny. Genes for several recessive characters were present in the test-chromosome, and consequently, in the absence of lethal mutations, each of these characters would be expected to be shown by one quarter of the F_2 generation following the outcross. However, if a lethal mutation was present, recessive characters linked to it would be absent.

Muller's first success with his lethal mutation counting techniques was the demonstration that a rise of temperature of 8°C produced a significant increase in the mutation rate. Using the accumulation method just described, 381 independent families kept at 19°C acquired a total of 12 lethal mutations in the test-chromosome in 16–17 generations occupying 11 months, and 359 lines kept at 27°C accumulated 31 lethals in the same chromosome in 18 generations during a period of 12 months. The mutation rates were therefore $\frac{12}{381 \times 16{\cdot}5} = 0{\cdot}19\%$ per chromosome per generation at 19°C, and $\frac{31}{359 \times 18} = 0{\cdot}48\%$ at 27°C. The difference was significant.

This demonstration represented a considerable achievement, because numerous previous attempts by many experimenters to bring about a significant alteration in mutation rate by changing the external conditions in various ways had met with failure. Muller had found mutation rates varying up to 10 times greater in some experiments than others from unknown causes but presumably primarily due either to differences in physiological condition or genotype. Unless the conditions were carefully controlled, as in Muller's experiment, such variation would obscure the effects of particular environmental factors.

A 2·5-fold increase of rate with an 8°C rise in temperature such as Muller found, pointed tentatively to mutation as a multimolecular chemical change, since such reactions give increases of this order ($Q_{10} = 2$–3).

§ 9.5 Mutations induced by radiations

Muller's second success with his techniques for studying mutation quantitatively was an outstanding one. Using the grandsonless-lineage

method, he showed (Muller 1927, 1928*b*) that *X*-rays enormously increased the rate of mutation. Males were irradiated and then crossed with females carrying the known lethal gene and the crossover suppressor in one of their *X*-chromosomes. Any daughters from this cross who failed to produce any sons must have had another recessive lethal gene in their other *X*-chromosome, the one derived from their irradiated father (see Table 9.1). To reduce the labour of searching for these grandsonless lineages, the dominant gene for Bar eye-shape was incorporated in the chromosome carrying the known lethal gene and the crossover suppressor. Only daughters with Bar

TABLE 9.1 Basis of Muller's grandsonless-lineage technique for detecting lethal mutations in the *X*-chromosome of *Drosophila melanogaster*.

Female Parent		Male Parent *X Y* Sperm	
		X^l	Y
$X_{ClB}X$	Eggs X_{ClB}	$X_{ClB}X^l$ Bar female	$X_{ClB}Y$ Dies
	Eggs X	$X\,X^l$ Normal female	$X\,Y$ Normal male

(*a*) Progeny of a female carrying in one of her *X*-chromosomes a crossover suppressor (*C*), a recessive lethal mutation (*l*), and the dominant gene for Bar eye-shape (*B*), crossed with a male in which a lethal mutation has occurred in the *X*-chromosome of the sperm. X = normal *X*-chromosome. X_{ClB} = *X*-chromosome with crossover suppressor (*C*), known lethal gene (*l*), and Bar mutant (*B*). X^l = *X*-chromosome with new recessive lethal mutation.

Female Parent		Male Parent *X Y* Sperm	
		X	Y
$X_{ClB}X^l$	Eggs X_{ClB}	$X_{ClB}X$ Bar female	$X_{ClB}Y$ Dies
	Eggs X^l	$X^l\,X$ Normal female	$X^l\,Y$ Dies

(*b*) Progeny of Bar female from (*a*) crossed with normal male. None of the sons survives.

eyes (and hence with the known lethal gene) were tested for their ability to produce sons. Non-lethal mutations in the *X*-chromosome can be detected at the same time as lethal ones, since the change of phenotype which they produce becomes evident in the grandsons of the irradiated males.

Muller found that male flies kept for 24 minutes at 16 cm from an X-ray tube (50 kV, 5 mA, 1 mm aluminium) gave 49 grandsonless lineages out of 676 tested, and others given the same irradiation for 48 minutes gave 89 such lineages out of 772 tested. The frequencies of lethal mutations in the *X*-chromosomes were therefore 7·2% and 11·5% respectively. Out of 198 control cultures, none were grandsonless, or, if a larger series of controls from other experiments is included, there were 5 lethal mutations in the *X*-chromosome of 6016 lineages tested, or 0·083%. The larger dose of X-rays thus gave a 140-fold increase in the mutation rate. As Muller had expected, the lethal mutations greatly outnumbered the non-lethal ones. Later work showed that, with both spontaneous and radiation-induced mutations, the proportion of lethal: non-lethal was about 10:1. Moreover, semi-lethal mutations giving much reduced viability (0·5—10% of normal) were rare: there were 4 with the 24-minute dose, and 12 with the 48-minute dose, or less than $\frac{1}{8}$ of the number of fully lethal mutations. Thus, the number of lethal mutations was reasonably clearly defined and likely to be a reliable guide to the total mutation-rate.

The visible mutations induced by X-rays included some at gene loci not previously known to have mutated, but others were allelic to, or apparently identical with, previously known spontaneous mutations such as white eye-colour. Some of the lethal mutations were allelic to visible ones, suggesting that the lethal ones did not differ in any fundamental way from non-lethal. There was great variety among the visible mutations and no suggestion whatever that X-rays were causing specific genes to mutate. All the indications were that X-ray induced mutations were essentially like spontaneous ones.

Muller's data suggested a direct proportionality between X-ray dose and number of mutations induced and this was later confirmed. Irrespective of the intensity at which the irradiation was administered, and also apparently of the wavelength, a dose of 1000 r gave about 2·5% of lethal mutations in the *X*-chromosome, 2000 r about 5%, and so on. There appeared to be no threshold dose, numerous small doses producing as many mutations as one large one of the same total size. The linear relationship between induced mutation rate and dosage pointed to a direct action of the radiation quanta or the released electrons upon the gene. However, different genes were found to mutate with significantly different frequencies, and this applied also to alleles. Thus, Timoféëf-Ressovsky (1934), in reviewing experimental work on mutation, summarised his own extensive studies with the white-eye alleles in *Drosophila*, in which he had found that many of the various allelic changes possible, including reciprocal changes, occurred with significantly different frequencies under standard X-ray treatment. These findings again paralleled the observations on spontaneous mutation, where different genes, including different alleles, showed diverse rates of change.

Almost simultaneously with Muller's discovery of the mutagenic action of X-rays on *Drosophila*, Stadler (1928) discovered that X-rays, and also the gamma-rays from radium, caused mutation in *Hordeum vulgare* (Barley). Germinating grains were irradiated, and following self-pollination of the resulting plants, the grains from each ear were isolated. On sowing these grains, it was found that a number of those derived from one ear showed an abnormality, while other ears produced only normal progeny. It was evident that following the irradiation, one or more shoots (tillers) were heterozygous for a recessive mutation, while the remaining shoots on the same plant did not carry the mutation. Altogether 48 different mutations for seedling characters, such as white, virescent, and yellow leaf-colour, were found in the progeny of the irradiated grains, and none in the progeny of untreated plants.

Within a short time after Muller's initial discovery, mutation had been induced by radiation in a wide range of plants and animals, and it was clear that the phenomenon was a general one. It was found that all ionizing radiations, and also ultraviolet light, caused mutation. In every instance, a whole range of kinds of mutations were produced, and there was no suggestion of specific action. The findings with X-rays appeared to be typical in most respects of the results also found with the other mutagenic radiations.

An important discovery about the process of mutation was made by Stadler (1930) with *Hordeum*, and by Muller (1930*b*) with *Drosophila*. Stadler X-rayed germinating barley grains at 10°C, 20°C, 30°C, 40°C and 50°C and found no significant differences in the mutation rate. Muller X-rayed *Drosophila* males at 8°C and 34°C, and using his grandsonless-lineage technique, found 22 lethal or semi-lethal mutations in 67 lineages (33%) in the former, and 32 out of 120 (27%) in the latter. A repetition of this experiment using a smaller X-ray dose but larger samples gave 33 mutations out of 403 (8·2%) for those treated at 8°C, and 13 mutations out of 208 (6·2%) for the 34°C irradiation. It was evident that in both *Hordeum* and *Drosophila* over a large range of temperature, the mutation-rate was approximately constant.

The deduction from these experiments was that X-ray-induced mutation could not be an ordinary multimolecular chemical reaction, because such reactions have a large temperature coefficient. Rather it appeared that a unimolecular reaction was involved. The appreciable effect of temperature on the spontaneous mutation rate in *Drosophila*, which Muller had earlier found (see § 9.4) could be reconciled with the X-ray temperature data if it were supposed that whatever caused the spontaneous mutations acted indirectly through the medium of a multimolecular, and hence temperature-sensitive, chemical reaction. Calculations showed that natural radioactivity was insufficient to account for more than about one five-hundredth to one thousandth of the spontaneous mutations occurring in *Drosophila*. Moreover, if the frequency of spontaneous mutations in a given time was essentially the same in all organisms, as dependence on natural radioactivity would presumably demand, long-lived organisms such as *Homo* would of course

accumulate much larger numbers of mutations per generation than would short-lived organisms such as *Drosophila*. For example, a lethal mutation in one *X*-chromosome out of every 1000 every 2–3 weeks (the *Drosophila* rate of 0·1% per generation) would imply that in 25 years about 1 in every 2 human *X*-chromosomes would acquire such a mutation. A consequence of this would be that the ratio of the sexes at birth would approximate to 2 girls : 1 boy instead of the equality (or slight preponderance of boys) that is found. It was evident that the spontaneous mutation rate was likely to be determined by the genotype and physiological condition of the organism, rather than by natural radioactivity, and that natural selection had probably acted to favour greater stability of the gene in long-lived organisms than in short. This conclusion implied that mutation rates could be influenced by chemical means.

§ 9.6 Mutations induced by chemicals

Despite the availability of Muller's highly efficient grandsonless-lineage technique for studying mutation quantitatively, it was 14 years after his discovery by this means of the mutagenic action of X-rays before clear evidence was obtained that mutations could be induced chemically. A variety of substances was tested by numerous experimenters, but the results were hardly statistically significant, until in 1941 Auerbach and Robson (1947) discovered that β-β'-dichloro-diethyl-sulphide (mustard-gas) was highly mutagenic.

A solution of mustard gas in cyclohexane was sprayed at 10-second intervals for a period up to 15 minutes into a stream of air passing over the flies. In the first experiment 1231 *X*-chromosomes were tested, that is to say, this number of daughters of treated males crossed to females carrying the known lethal gene was tested for their ability to produce sons. There were 90, or 7·3%, of these grandsonless lineages compared with 3 out of 1216, or 0·25%, in the untreated control. The treatment also gave rise to 11 semi-lethal mutations, whereas there was none in the control. A frequency of 7·3% of recessive sex-linked lethal mutations was comparable to that produced by 3000 r of X-rays, and was clearly highly significant.

Chemical mutagenesis was discovered independently by Rapoport (1946), who found that a solution of formaldehyde when mixed with the food of *Drosophila* in sublethal concentrations, gave 5·9% of sex-linked lethal mutations.

Following these initial discoveries with mustard-gas and formaldehyde, a wide range of chemicals was soon found to cause mutation, for example, urethane (ethyl carbamate), nitrogen mustard, manganous chloride and hydrogen peroxide. Numerous organisms were found to show mutations when treated with these substances, and their mutagenic action appeared to apply generally. The effect of these chemicals was similar to the effects of mutagenic radiations, that is to say, each produced a wide range of kinds of mutations and there was no evidence for specific action on particular genes.

§ 9.7 Structural changes in chromosomes

Before considering the significance of knowledge about mutation for the theory of the gene, another feature of mutagens must be considered. Muller (1927) discovered not only that X-rays caused mutation but also that they caused structural changes in linkage groups. This was revealed when linkage studies were made following irradiation. The recombination frequencies of genes were frequently less or more than had been found previously, suggesting that portions of hereditary material had been removed or added. Moreover, the sequence of genes was sometimes reversed, implying that a section had been turned round. In still other instances, genes showed linkage in inheritance where hitherto they had belonged to different linkage groups, and conversely, genes previously linked now showed independent inheritance. It was evident that part of one linkage group had become attached to another. Changes of all these kinds had been known previously, but they had appeared only very rarely. It seemed as if, as with mutation, X-rays were increasing the frequency of occurrence of phenomena which also occurred in their absence. It had been anticipated for some years that X-rays would be likely to cause chromosome breakage, but a remarkable feature of Muller's discovery was the frequency with which the hereditary material appeared to be joined up in new ways. Thus, Muller and Altenburg (1930) found no less than 117 instances of transfer of part of one linkage group to another in 883 lineages tested following a heavy dose of X-rays applied to *Drosophila* males.

Muller and Painter (1929) and Dobzhansky (1929) confirmed that structural changes in *Drosophila* linkage groups, following X-irradiation, were associated with corresponding changes in the chromosomes. Their work finally established that the order of the genes on the linkage map is the same as their actual physical order in the chromosome, although the relative distances often did not correspond. A large section of linkage map removed or transferred might correspond to only a small section of the chromosome, or the converse might be true. It was evident that the scale of the linkage maps varied greatly from region to region, when compared with the physical chromosome, crossing-over being more frequent in some regions than others.

Further confirmation that structural changes in *Drosophila* linkage groups involved rearrangement of the material of the chromosomes was obtained from study of the giant chromosomes in the nuclei of the salivary glands of the mature larvae. These chromosomes had been known in some other Diptera for over 50 years, before Painter (1934) discovered their value in genetics when he showed that structural changes in *Drosophila* linkage groups can be correlated with changes in the sequence of transverse discs or bands in these chromosomes. Corresponding parts of homologous chromosomes are closely paired in these salivary gland nuclei, and all the chromosomes are held together at their centromeres, the paired chromosome arms, of which there are five in *D. melanogaster* (the very short fourth chromosome apart), being cylindrical in shape, about 5 μ in diameter, and radiating for a distance of about 250 μ if straightened (Fig. 9.1 and Pl. 1(f)). By contrast, in the

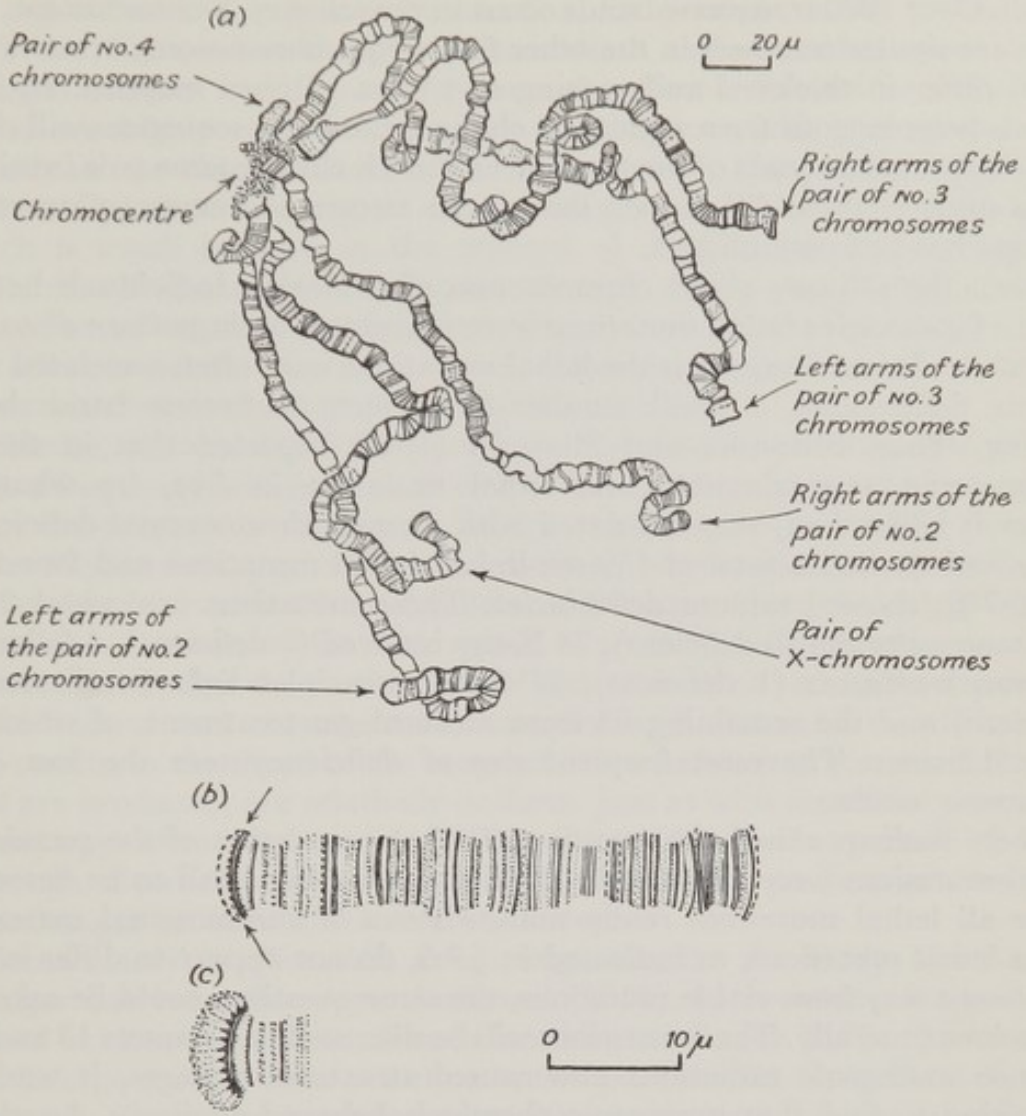


FIGURE 9.1 Drawings of the giant chromosomes found in the cell nuclei of the salivary glands of Diptera.

(*a*) The complete set of salivary gland chromosomes of *Drosophila melanogaster* (from Painter, 1934). The two homologous chromosomes of each kind are fused together throughout their length, and all the chromosomes are held together in the region of their centromeres, forming the chromocentre.

(*b*) The no. 4 chromosome-pair of *Chironomus tentans* (from Beermann, 1961). The arrows show the position of the gene concerned with the secretion of granules in particular cells of the salivary glands. In *C. tentans* this gene occurs in a mutant form such that no granules are produced.

(*c*) The left-hand end of the no. 4 chromosome-pair of *C. pallidivittatus* showing a puff formed at the position of the gene for granule-secretion.

reproductive cells at meiosis the chromosome arms measure about 2 μ in length. Over 1000 transverse bands occur in the salivary *X*-chromosome, and there are similar numbers in the other four major chromosome arms. These bands differ in thickness and spacing and have all been mapped. In individuals heterozygous for a structural change, the band sequences will differ in the appropriate parts of the two halves of each chromosome pair involved. From study of these differences, the precise nature of numerous structural changes has been established.

When the salivary gland chromosomes of *Drosophila* individuals heterozygous for recessive lethal mutations were examined, an important discovery was made. It was found that the lethal mutations were often associated with minute deficiencies, a small number of adjacent transverse bands being missing. Thus, Slizynska and Slizynski (1947) reported that in the *X*-chromosome approximately one lethal mutation in five, by whatever means it had arisen, was associated with a small chromosomal deficiency. They had studied a total of 159 sex-linked lethal mutations and found 33, or 20·7%, showed minute deficiencies. These mutations comprised 27 of spontaneous origin (6 deficient), 24 X-ray induced (5 deficient), 4 following neutron treatment (1 deficient), 21 from ultraviolet light irradiation (5 deficient) and the remaining 83 from mustard-gas treatment, of which 16 were deficient. The most frequent size of deficiency was the loss of 2 transverse bands.

These findings raised the question of what proportion of the remaining lethal mutations were associated with deficiencies too small to be detected. Were all lethal mutations really minute losses of chromosomal material? Since lethal mutations, as indicated in § 9.5, do not appear to differ in any significant way from viable mutations, the same questions could be asked of mutations generally. These questions will be discussed in Chapters 13 and 14.

Since mutagenic radiations also caused structural changes, it was not surprising to find that mutagenic chemicals behaved similarly. Auerbach and Robson (1947) from study of genetic linkage in *Drosophila* following treatment of the flies with mustard-gas, and Oehlkers (1943) from study of *Oenothera* chromosomes at meiosis after keeping cut inflorescences in a solution of urethane (ethyl carbamate), were the first to demonstrate the induction of structural changes by chemical means. A wide range of chemicals has since been found to have this property of causing chromosome aberrations.

§ 9.8 The mechanism of induction of structural changes

Thoday and Read (1947) found that the effect of X-rays in causing structural changes in chromosomes was much influenced by the oxygen concentration at the time of irradiation. Root-tips of *Vicia faba* irradiated in the absence of oxygen gave only about one third of the aberration frequency that the same dose gave in oxygen. Kihlman (1961), in reviewing biochemical knowledge concerning the induction of structural changes, classified agents causing aberrations into three main categories.

First, some agents induce structural changes with a frequency which does not depend on the oxygen concentration, for example, alpha particles and ultraviolet light, where the effect is immediate, and alkylating agents such as nitrogen mustard and diepoxides, where the effect is delayed for some hours. These are highly reactive chemicals which presumably act more or less directly on the chromosomes, as the radiations are also presumed to do.

Secondly, there are agents which cause aberrations with a frequency which is much reduced in the absence of oxygen, but is unaffected by inhibitors of respiration. Examples of such agents are X-rays, with an immediate effect, and visible light in the presence of acridine orange, where the effect is delayed for some hours. Kihlman suggested that the oxygen reacts with free radicals produced by the radiations.

Thirdly, certain chemicals have been found to cause structural changes with a frequency which is much reduced, either by the removal of oxygen, or by the presence of respiratory inhibitors such as sodium azide. Examples are methylated oxypurines such as caffeine, where the effect is immediate, and maleic hydrazide where it is delayed for some hours. Evidently aerobic respiration is necessary for these agents to produce their effects.

It is evident that the mechanisms of induction of chromosome aberrations by radiations and chemicals are complex and diverse. Nevertheless, the end-results, in the shape of the range of different kinds of structural changes that are produced, are relatively uniform. Just as with mutation, mutagenic agents appear to produce the whole range of kinds of structural changes, although ultraviolet light has usually been found to produce relatively few interchanges between different chromosomes.

Second only to *Drosophila* as an organism for the study of structural changes has been *Zea mays*. Anderson (1936), in reviewing his own and others' work on such aberrations induced by irradiation in *Zea* chromosomes and linkage groups, drew attention to two peculiarities about these changes. First, all alterations involving two chromosomes or linkage groups were reciprocal, that is to say, if a piece of one chromosome was transferred to another, then a piece of the latter was also transferred to the former, and furthermore the rejoining was always at the points of breakage, never at the normal ends of the transferred segments. Secondly, all changes involving only one chromosome, such as inversions, duplications, deficiencies, and the production of ring-shaped chromosomes, involved interstitial segments. Deficiencies which appeared to be terminally placed, that is, loss of the end of a chromosome, were found on careful study to be in fact interstitial in position, the end segment being attached to the broken end of the main part of the chromosome.* It was evident that all these changes involved two points of breakage, followed by rejoining in new ways, and moreover, the normal ends of the chromosomes did not take part in this rejoining. These observations resembled those made with *Drosophila* referred to in § 9.7.

In order to account for these discoveries two hypotheses have been put forward. According to one, the initial event is chromosome breakage. On this

* Some exceptions to this are now known: see for example § 9.11.

hypothesis, it is necessary, in addition, to postulate that broken ends can rejoin, either in the original way or in new ways, but that normal chromosome ends cannot do so. According to the alternative hypothesis, the mechanism of induction of structural changes is by a process of non-homologous crossing-over or exchange, which is postulated as occurring either between different chromosomes, or between different parts of the same chromosome. This idea was first proposed by Belling (1927) with reference to an interchange of segments between non-homologous chromosomes, which he and Blakeslee had discovered in *Datura stramonium*. On this theory, the failure of normal chromosome ends to take part in rejoining is accounted for, without the necessity of postulating that these ends have special properties which prevent them from doing so.

Studies on the relation between the frequency of interchanges between different chromosomes and the dose of *X*- or gamma-rays showed that the frequency increased approximately as the square of the dose. A square law would be expected if two independent events were required to bring about the interchanges. These results were at first taken as evidence in support of the breakage-and-reunion theory. It was assumed that the two independent events which the square law demanded were the breaking of the two chromosomes by separate ionisation tracks. Furthermore, it was assumed that, on the other hypothesis, the first step in non-homologous crossing-over would be the making contact between two chromosome segments, and that a single ionisation-event in the vicinity of the paired segments would cause the process of exchange to occur. On this assumption, non-homologous crossing-over would give rise to a linear relationship between radiation dose and interchange frequency. Since the relationship was non-linear it was argued that the theory of non-homologous crossing-over was untenable. However, Revell (1955) pointed out that the square law does not necessarily invalidate the hypothesis of non-homologous crossing-over if one assumes that the two separate primary events are the triggering off, independently in each chromosome, of a series of changes leading to mutual crossing-over.

If the effect of radiations (and of mutagenic chemicals also, since their effects are similar) is to set in train a series of changes leading to non-homologous crossing-over, it is reasonable to suppose that this process will have features in common with normal crossing-over between homologous chromosomes at meiosis, and in particular that it will occur after the chromosomes have divided into chromatids, and that it will involve only one chromatid from each chromosome (or from each of the two participating segments of the same chromosome). Anderson (1936) made these assumptions for, having established that normal crossing-over occurs at the four-strand stage (see § 8.6), he favoured a similar situation for the hypothetical non-homologous process. The consequences of crossing-over between different parts of the same chromosome depend (a) on whether the bending back of the chromosome to bring non-homologous parts together is in the form of a letter U, or of a coil, and (b) on whether the chromatids involved are identical or sisters (see Fig. 9.2). Anderson pointed out that crossing-over between different parts of the same chromatid would give rise either to an interstitial

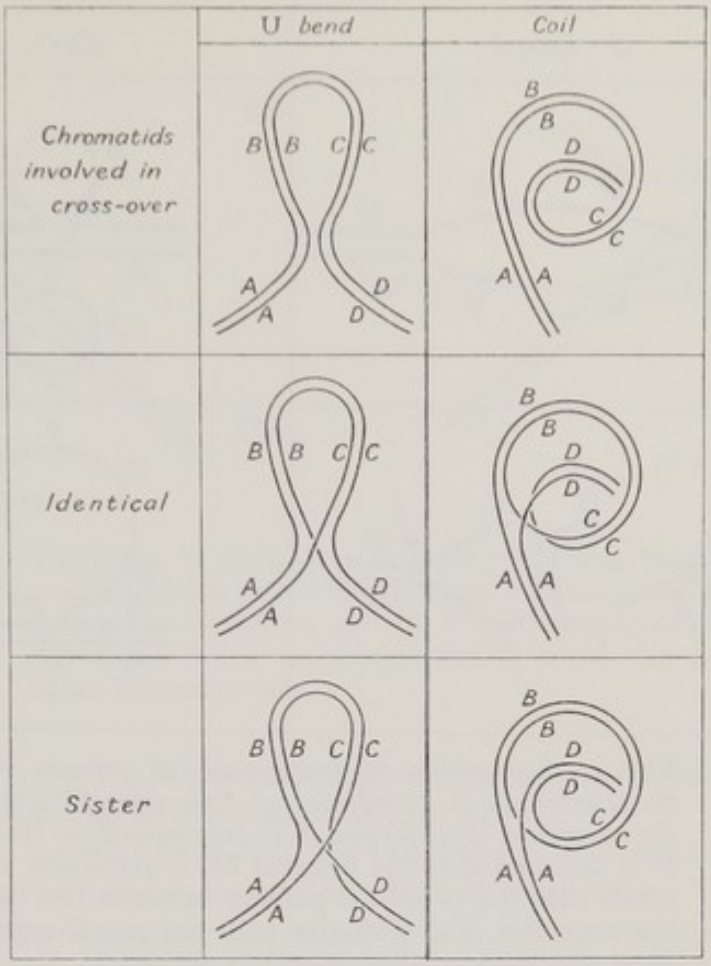


FIGURE 9.2 The four ways in which non-homologous crossing-over at the four-strand stage may occur between different parts of the same chromosome. The lines represent chromatids and the letters *A–D* denote different regions of the chromosome segment.

inversion (Fig. 9.3(*a*)) or to an interstitial deficiency, the missing segment forming a separate ring-shaped chromatid (Fig. 9.3(*b*)). The other chromatid would be normal, since it would not have taken part in the exchange. If the crossing-over was between different parts of sister chromatids, either a pair of hairpin-shaped segments, each with a duplication and a deficiency, would be formed (Fig. 9.3 (*c*)), or there would be a duplication of a segment in one chromatid and a corresponding deficiency in the other one (Fig. 9.3(*d*)).

On the alternative breakage-and-reunion theory, the configurations just described are also possible, but a simple union of sister chromatids would be expected much more frequently, following breakage of both chromatids at approximately corresponding positions. This would give rise to a pair of hairpin-shaped segments (Fig. 9.4(*a*)) similar to those derived from non-homologous crossing-over (Fig. 9.3(*c*)) but differing from them in certain

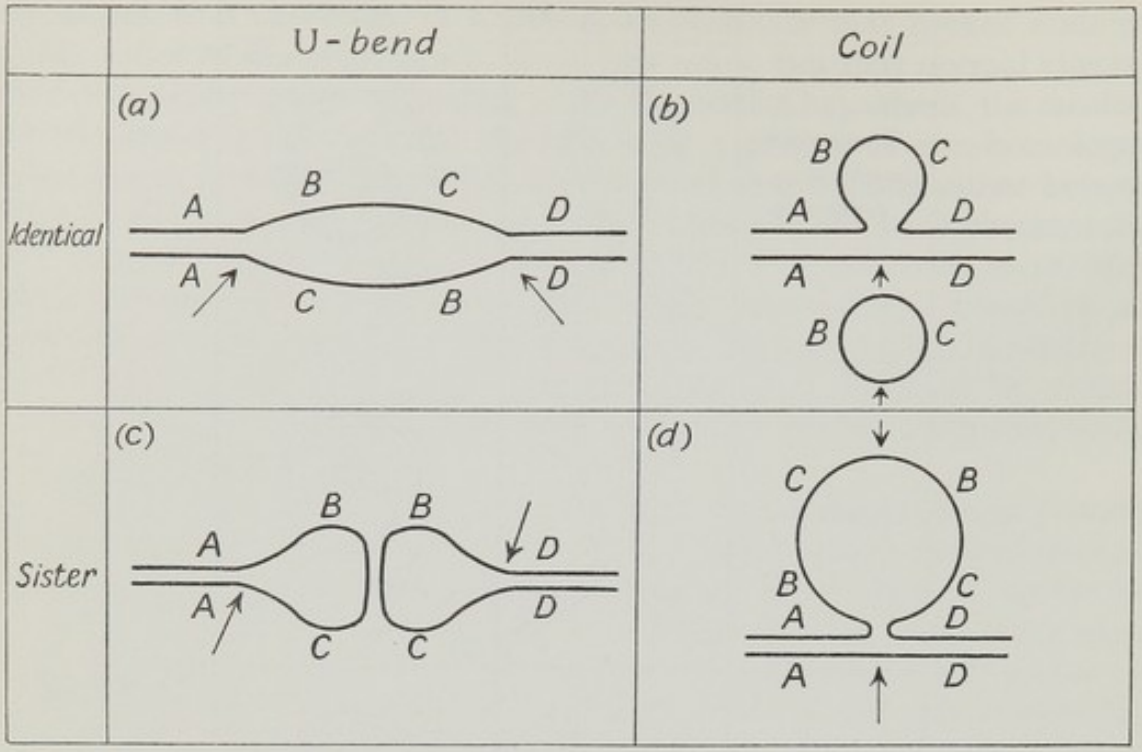


FIGURE 9.3 The configurations at metaphase of mitosis resulting from non-homologous crossing-over between different parts of a chromosome. The letters *A–D* correspond to those in Fig. 9.2. It is assumed that the interval *BC* is relatively short, with the result that the prophase pairing between the two *B* segments has been lost, and similarly with the two *C* segments. Arrows show the points where union has occurred.

respects. The differences are indicated by the lettering in the diagrams and by the arrows which show the points of reunion.

In *Vicia faba* root-tip cells at metaphase, following *X*-irradiation in the preceding resting-stage, Revell found that many of the pairs of hairpin-shaped segments resulting from the irradiation showed constrictions at the positions of the arrows in Fig. 9.3(*c*), but not at the positions in Fig. 9.4(*a*). The constrictions were interpreted as showing the points of crossing-over, since similar constrictions were often seen at the position where rejoining had

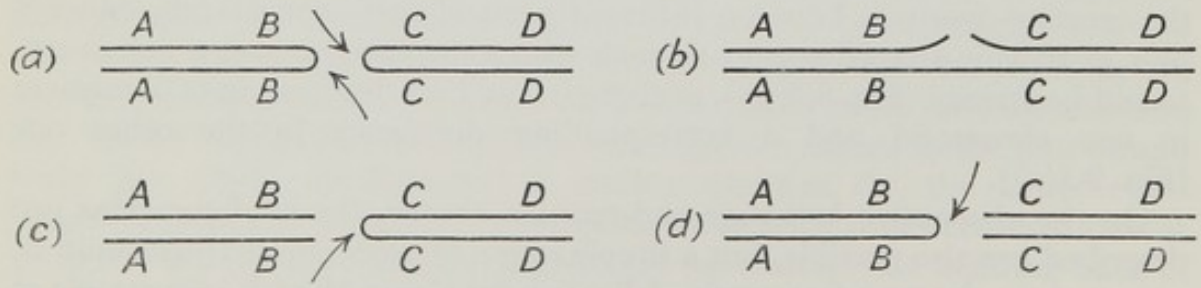


FIGURE 9.4 The types of chromosomal aberration expected on the breakage-and-reunion hypothesis (*a*) if all broken ends show union, and (*b–d*) if two broken ends fail to show union. Arrows indicate the points where union has occurred.

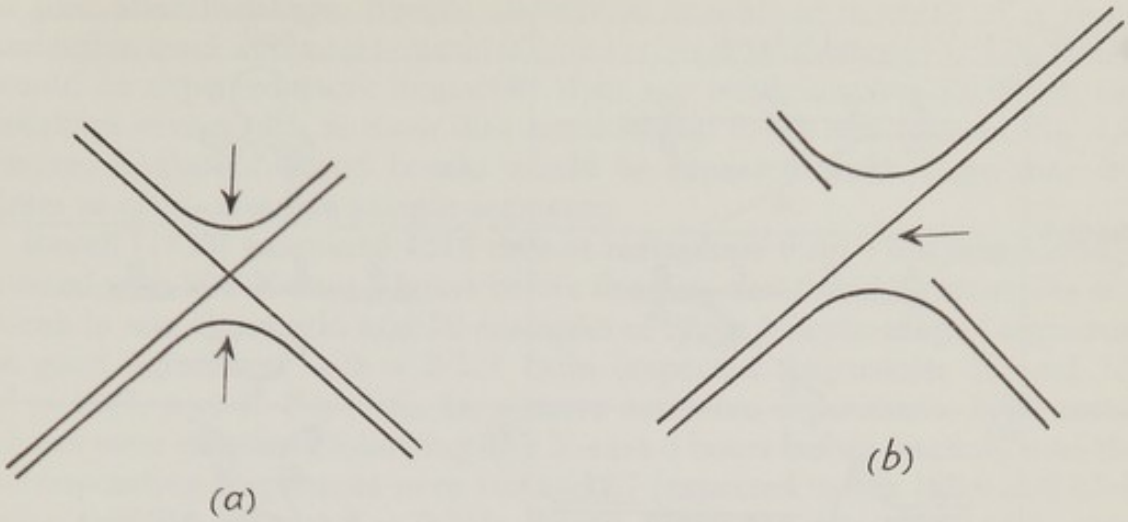


FIGURE 9.5 The types of aberration expected following non-homologous crossing-over between two different chromosomes (*a*) following a complete exchange, and (*b*) if the free ends of one crossover chromatid failed to unite. Arrows show the points where union has occurred.

evidently occurred when different chromosomes had undergone interchange, that is, at the positions of the arrows in Fig. 9.5(*a*).

Furthermore, Revell found that the two hairpin segments of a pair were usually in close proximity to one another at metaphase. This would be expected if their construction was as in Fig. 9.3(*c*) where the two homologous segments labelled *B* would be expected to remain associated until metaphase, and similarly with those labelled *C*. If the construction of the hairpin segments was as in Fig. 9.4(*a*), they would be expected to have drifted apart by metaphase.

Anderson considered the genetic consequences of these structural changes, supposedly resulting from non-homologous crossing-over at the four-strand stage between different parts of the same chromosome, and pointed out that quantitative predictions capable of experimental testing would be complicated by viability phenomena, since chromosome segments lacking a centromere are soon lost from a cell, and such deficiencies often cause inviability, particularly to pollen-grains. Revell, on the other hand, has been concerned with the immediate cytological consequences, before differential viabilities take effect. Accurate quantitative predictions can then be made. Thus, Revell (1959) assumed that the two kinds of bending (U-shaped and coil) occurred with equal frequency, and that the exchanges involved either identical or sister strands equally often. The four configurations shown in Fig. 9.3 are then expected with equal frequency, and theoretically, by examining chromosomes fixed a few hours after irradiation, it should be possible to observe and count these aberrations. This should allow a direct test of the exchange hypothesis, since on the alternative breakage-and-reunion theory the pair of hairpin segments (Fig. 9.4(*a*), but resembling Fig. 9.3(*c*)) would be expected with greater frequency than the configurations shown in Fig. 9.3, for the reasons given above.

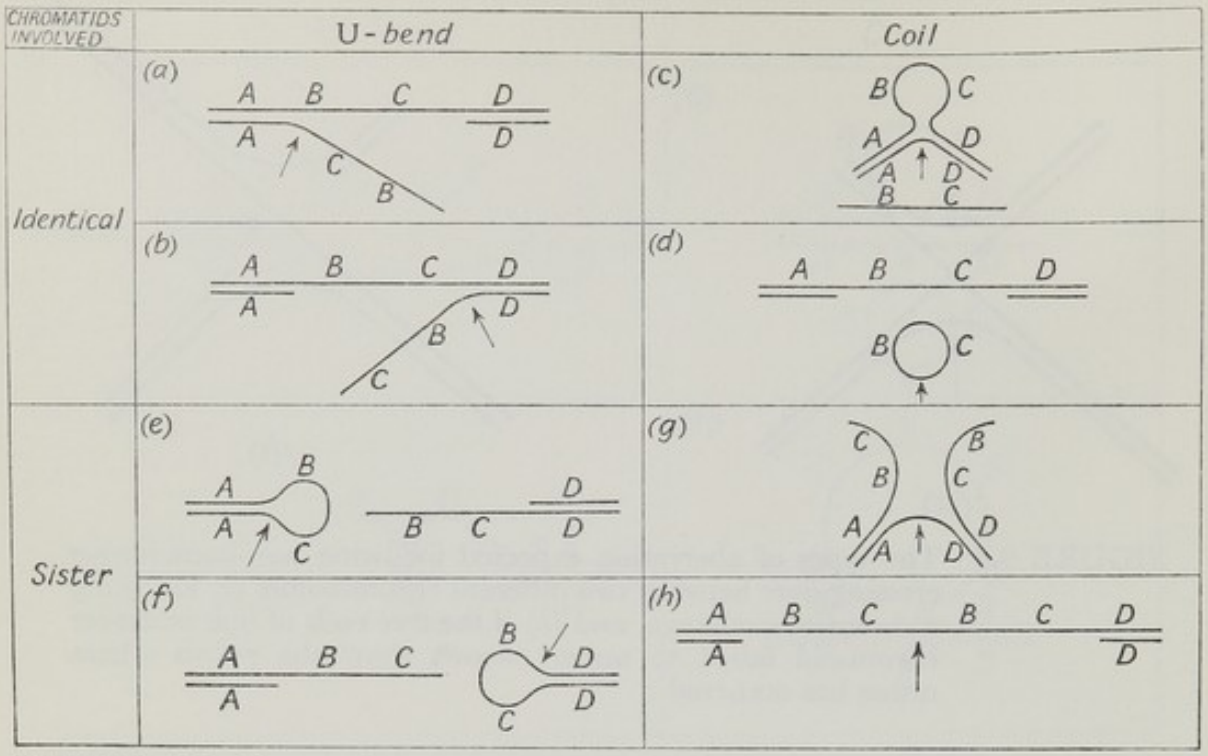


FIGURE 9.6 The configurations expected at metaphase of mitosis as a result of 'half crossing-over' between non-homologous segments of a chromosome. The letters *A–D* correspond to those in Figs. 9.2 and 9.3. Arrows show the points where union has occurred.

In practice, if the segment BC between the points of supposed non-homologous exchange is short, as appears usual, the configurations in Fig. 9.3(*a*), (*b*) and (*d*), often cannot be identified with certainty, and even the ring-chromosome (Fig. 9.3(*b*)) may be overlooked if it is small. Only the pair of hairpin segments (Fig. 9.3(*c*)) is readily recognised. Revell thus found that, at least in *Vicia faba* root-tips, a straightforward quantitative test of the hypothesis of non-homologous crossing-over could not be made. However, he suggested that in a minority of instances (7–10% in his experiments described below) the induced process of crossing-over was not completed, with the result that, although one cross-over chromatid was formed, the free ends of the reciprocal one failed to unite. Such a 'half cross-over' would be directly recognisable as a 'half-chiasma' when different chromosomes were involved (Fig. 9.5(*b*)), but the consequences when different regions of the same chromosome were concerned are diverse and are shown in Fig. 9.6. There are 8 alternatives depending on which way the chromosome bends (U or coil), which strands undergo exchange (identical or sister), and which fail to unite. The configurations shown in Fig. 9.6(*a*), (*b*), (*d*), (*g*) and (*h*) will appear as a break in one chromatid, those in Fig. 9.6(*e*) and (*f*) will appear as single hairpin-shaped segments, while that in Fig. 9.6(*c*) will appear more or less normal. Hence, on the assumption that the 8 alternatives occur with equal frequency, the occurrence of a break in one chromatid is expected to be 2·5 times as frequent as the occurrence of single hairpin segments. On the other hand, on the breakage-and-reunion theory, configurations with free ends would be the direct result of a failure

to join after breakage. Simple chromatid breaks, as a result of a single ionization track and a subsequent failure to rejoin, as illustrated in Fig. 9.4(*b*), would be expected more frequently than any configurations involving two breakage events such as those illustrated in Fig. 9.4(*c*) and (*d*) and Fig. 9.6. Hence, single chromatid breaks would be expected much more than 2·5 times as often as single hairpin segments.

Revell (1959) examined 1513 cells at metaphase from *Vicia faba* root-tips treated with 50 r X-rays 5 hours before fixation, and found 32 examples of a break in one chromatid and 10 examples of single hairpin-shaped segments, in good agreement with a 2·5:1 ratio (expected frequencies 30 and 12, $\chi^2 = 0{\cdot}47$, $n = 1$, $P = 0{\cdot}5$). In a more extensive experiment, 5000 metaphases were examined following 65 r X-rays 5 hours before fixation, and the corresponding frequencies were 169 and 67 (expected values 168·6 and 67·4, $\chi^2 = 0{\cdot}0038$, $n = 1$, $P = 0{\cdot}95$). Revell found no less than 4155 non-staining gaps or 'false chromatid breaks' in the 5000 metaphases, many or all of which would formerly have been scored as breaks, before it was recognised that a thread so fine as to be beyond the resolution of the microscope may join chromatid segments.

These results, taken in conjunction with the qualitative evidence given above, provide strong support for the hypothesis of non-homologous crossing-over as an explanation of the origin of structural changes induced by radiations. Furthermore, Revell has found that hairpin-shaped segments and chromatid breaks increase in frequency in proportion to the X-ray dose raised to power 1·5. This conflicts with the predictions of the breakage-and-reunion hypothesis, where chromatid breaks would be expected to show a linear relation with dose, but is in agreement with the exchange hypothesis, assuming that non-homologous crossing-over between different regions of the same chromosome is sometimes due to a single ionization track and sometimes to two.

According to a recent hypothesis, the initial event in crossing-over is breakage of one of two longitudinally arranged sub-units of each participating chromatid, followed by association between these sub-units (see Chapter 16). There are several points of agreement between this postulated mechanism and the requirements of the Anderson-Revell chromatid-exchange hypothesis for the origin of structural changes (see § 17.5).

§ 9.9 The hypothesis of the chromosome as a string of beads

The classical concept of the gene, which grew up as soon as the chromosome theory was accepted, was to liken the chromosome to a string of beads, each bead representing a different gene. This idea incorporated all the salient features about genes, namely, that they behaved in inheritance as discrete particles which appeared not to influence one another, that they were linearly arranged in the chromosome, and that crossing-over between homologous chromosomes at meiosis apparently occurred between them.

The three primary ways of defining the gene, that is, in terms of its specific effect on a particular character or group of characters, in terms of its

recombination with neighbouring genes as a result of crossing-over, and in terms of its change to a different allele as a result of mutation, were thought to represent different aspects of the same structure. Thus, the gene was considered to be a specific molecule or aggregate of molecules making up the bead, which in some way controlled the development of a particular character in the organism. Crossing-over was imagined as a process of mechanical exchange which could cause breakage between corresponding beads in homologous chromosomes followed by rejoining in new ways, but which necessarily left the beads themselves intact. Mutation was thought of as an abrupt change in the molecular structure of the gene of a kind which was stable and was copied when the molecule was duplicated, and thereby inherited, and which led to a change in the appropriate character of the organism.

Multiple alleles could also be incorporated into the bead hypothesis, since it was only to be expected that the molecular structure of a gene could be changed in many different ways. Structural changes would represent abnormal rearrangements of bead sequence, or the duplication or omission of some of them. Two other observations appeared to reinforce the bead concept. Firstly, the sequence of the genes in the chromosome seemed to be a random, fortuitous one, such that neighbouring genes were presumed to be concerned with totally unrelated characters in the organism. This heterogeneity supported the idea that neighbouring genes in no way influenced one another, and that their proximity was merely a consequence of the favouring by natural selection of a stringing together of the hereditary units as a more efficient means of distributing them with precision between daughter-cells at cell-division. Secondly, the structure of a chromosome as seen at the leptotene stage of meiosis had itself been likened to a string of beads. Belling (1928*b*) went so far as to postulate that each chromomere represented a specific gene, and others made a similar suggestion about the discs or bands in salivary gland chromosomes. Without going as far as this, many saw in the visible heterogeneity of the chromosome support for the bead hypothesis.

On the other hand, Raffel and Muller (1940) pointed out that although it was often assumed that the lines of demarcation between genes, as defined by crossing-over, breakage, mutation, function, and reproduction, would coincide with one another, there was no evidence for this, and the assumption had only a doubtful theoretical basis. Moreover, it was only an assumption that the lines of demarcation of the units defined by each of these criteria were necessarily invariable, non-overlapping or even well-defined. In other words, there was no evidence that the hereditary material was really subdivided into intra- and inter-genic components, such as the bead idea implied. The gene was recognised at the functional level by its effect on the development of the organism, but it was not a necessary consequence of this that genes were differentiated from one another physically: the beads and the thread connecting them might both be made of the same material. Goldschmidt (1938) had taken this idea to its logical conclusion when he suggested that it was the chromosome, which he thought of as a large chain

molecule, which was the hereditary unit, that mutations were changes in this molecule at specific points, and that these changes disturbed the normal interplay of catalysed reactions. He considered that each point in the chain had a definite meaning in the chemical properties of the whole, as with all molecules, but that the unmutated (or wild-type) gene was merely a theoretical concept. That is to say, he regarded the normal (wild-type) condition as controlled by the whole chain as a unit, without the separate existence of individual genes.

Few geneticists were willing to go as far as Goldschmidt, with whom the string-of-beads idea had become a string without beads. Nevertheless, it was recognised that there was no evidence that the units of function, of recombination, and of mutation were co-extensive, such as the bead hypothesis assumed. Indeed, evidence against this hypothesis was accumulating.

§ 9.10 Evidence against the bead hypothesis: the position effect

The bead hypothesis suffered its first setback when Sturtevant (1925) made a most unexpected discovery. The dominant sex-linked gene called Bar, which causes a narrow eye in *Drosophila melanogaster* instead of the normal round eye, was unusual in that it appeared to mutate with comparatively high frequency. Zeleny (1921) found that, out of 85,008 progeny of homozygous Bar-eyed females crossed with normal males, 52 (or approximately 1 in every 1600) showed reversion to the normal round eye, and 3 (or approximately 1 in every 28,400) showed mutation to a more extreme form dominant to Bar, which he called Ultra-bar. Ultra-bar itself showed similar changes.

Sturtevant and Morgan (1923) found that these changes were confined to the female, and moreover were not true mutations but were due to crossing-over. They studied the progeny of homozygous Bar-eyed females which were heterozygous for the recessive genes for *forked* bristles and for *fused* wing-veins. These genes are placed 0·2 and 2·5 units, respectively, away from Bar on either side. It was found that, whenever a reversion from Bar to normal eye-shape occurred there was crossing-over between the loci of the 'marker' genes *forked* and *fused*. Sturtevant (1925) found that this also applied to the origin of Ultra-bar from Bar. Some of his data are given in Table 9.2. He concluded that the normal round and the more extreme Ultra-bar eye characters arose from Bar (*B*) through crossing-over occurring obliquely so as to give two Bar alleles (*BB*) in one chromosome (Ultra-bar) and none in the other (round). The round-eyed flies arising from homozygous Bar through crossing-over were entirely normal, both in appearance and in their progeny. The lower frequency with which Ultra-bar arose compared with round was attributed partly to oversight, since Ultra-bar is not very dissimilar from Bar, and partly to a lower viability.

Sturtevant compared the appearance of the eyes in the various Bar genotypes, and made the surprising discovery that the heterozygote of

Ultra-bar and round $\left(\frac{BB}{\cdot}\right)$ had narrower eyes (mean number of facets 45*) than the Bar homozygote $\left(\frac{B}{B}\right)$ (mean number of facets 68*), although each fly contained the same two Bar alleles. He concluded that two alleles were more effective in narrowing the eye when they lay in the same chromosome than when there was one in each chromosome. Muller, Prokofyeva and Kossikov (1936) and, independently, Bridges (1936) carried the analysis a

TABLE 9.2 Data of Sturtevant (1925) for the progeny of Bar-eyed (*B*) *Drosophila melanogaster* with marker genes *forked* bristles (*f*) and *fused* wing-veins (*fu*) on either side of the Bar locus. Plus signs indicate normal alleles.

Parents: $\frac{+\ B\ +}{f\ B\ fu} \times f\ B\ fu.$ F_1 Progeny:—

Eye-shape	Numbers of Various Genotypes for Outside Markers				
	Parental		Recombinant		Total
	+ +	*f fu*	+ *fu*	*f* +	
Homozygous Bar†	10,631	7,909	187	264	18,991
Heterozygous Bar† (females)	0	0	0	2	2
Normal round (males)	1‡	0	2	1	4
Ultra-bar	0	0	0	2	2

† Homozygous Bar and the heterozygote of Bar and normal can be distinguished by the eye shape, which is more extreme in the homozygote.

‡ Thought to be a contaminant.

stage further when they showed that the Bar phenotype was not due to a simple gene mutation but to a structural change. Study of the salivary gland chromosomes revealed that Bar-eyed flies had a chromosome segment (*S*) containing 6 transverse bands present in duplicate. The repeated section was adjacent to the original and in the same (not the reversed) sequence, giving the configuration *SS*. Ultra-bar flies had the same segment in triplicate (*SSS*), while the normal flies arising from the Bar-eyed ones through crossing-over had normal salivary gland chromosomes with the segment represented only once. Sturtevant and Morgan's discovery of oblique crossing-over was now explained on the supposition that the left-hand *S* segment in one chromosome of homozygous Bar-eyed flies occasionally paired at meiosis with the right-hand *S* segment in the homologous chromosome $\left(\frac{SS}{SS}\right)$. A cross-over

* Facet number was found to be smaller the higher the temperature during development over the range 15–30°C. These frequencies refer to flies grown at 25°C.

in these paired segments would then give a gamete with the segment in triplicate (*SSS*) and another in which the segment was represented only once (*S*). This staggered pairing evidently happened in either direction (the right-hand segment on one homologue with the left-hand segment of the other, and *vice versa*), because the round-eyed flies derived from Bar could have either of the two complementary crossover genotypes for the marker genes, that is, either non-*forked fused* or *forked* non-*fused* in Sturtevant's data in Table 9.2. Sturtevant's earlier deduction that two Bar alleles in one chromosome were more effective than one in each, had to be replaced by the notion that the particular segment, *S*, if present in triplicate in one chromosome and represented once in the other $\left(\frac{SSS}{S}\right)$ resulted in a narrower eye than the same segment in duplicate in both chromosomes $\left(\frac{SS}{SS}\right)$.

It was evident that the appearance of an organism may be modified merely by altering the arrangement of the hereditary material in the chromosomes, without mutation and without loss or gain in the quantity of genetic material. On the bead hypothesis, the only way to accommodate Sturtevant's discovery of the effect of position on the phenotype was to postulate that the beads were not the isolated structures that had been supposed, but that neighbouring beads (genes) could either influence one another, or their immediate products of activity could interact, thereby modifying the particular characters of the organism which they controlled. Although this initial example of an alteration of the phenotype through rearranging the hereditary material referred to an abnormal situation involving duplicated and triplicated segments, it was later established that the principle which it illustrates is of wide application and fundamental importance. It is discussed in § 13.3 and § 18.2.

§ 9.11 Evidence against the bead hypothesis: recombination between alleles

Work on a series of bristle mutants called *scute* of *Drosophila melanogaster* by a number of authors and extending over many years had suggested that the units of function, recombination and mutation might not correspond (cf. Raffel and Muller, 1940). More specific evidence for such lack of correspondence was obtained when Oliver (1940) discovered recombination in *D. melanogaster* between two allelic eye-character mutants called *lozenge*. The mutant alleles were both recessive to normal and sex-linked, and they gave rise in the homozygote to a reduction in the pigmentation and other effects on the eye. Oliver found that females heterozygous for the alleles, that is, with one in one chromosome and the other in the homologue, when crossed with males carrying either allele, gave small numbers of progeny with normal eyes, and, furthermore, these reversions were always associated with crossing-over in the vicinity of the alleles. This remarkable finding recalled Sturtevant and Morgan's discovery with Bar eye, but differed from it in an important respect: the normal-eyed progeny from the lozenge-eyed

parents were associated with one particular crossover genotype, whereas with Bar, as indicated above, they were associated with either crossover combination of the marker genes.

Lewis (1941) made a similar discovery with two *star* eye-character alleles in the second chromosome. These alleles, one dominant (*S*) and the other recessive (*s*) to normal, caused a reduction in size and an increase in roughness of the eye-surface. Heterozygotes having *S* in one chromosome and *s* in the other had *star* eyes as expected with alleles. Females heterozygous for these two alleles and also for the recessive genes *arista-less* (*al*) and *held-out* wings (*ho*), which are closely linked on either side of the *star* locus, were crossed with star-eyed males and gave rise to 4 normal-eyed progeny among 31,106 star-eyed. The female parents had the genotype $\frac{al\ S\ ho}{+\ s\ +}$ (the + sign indicates the normal alleles), and all 4 normal-eyed progeny had evidently developed from eggs of constitution $+\ +\ ho$, or in other words they were crossovers for the marker genes on either side, having the normal allele of *arista-less* together with the *held-out* mutation. In another experiment using a stock with increased recombination in this region, there were 12 normal-eyed flies from the same cross among 26,370 star-eyed, and again the normals were all non-*aristaless* and *held-out*.

It was evident that the sites of the two star alleles were not quite identical, and it appeared as if crossing-over occasionally took place between them. That the normal-eyed flies carried the mutation *held-out* but not *arista-less* implied that the dominant star allele was slightly nearer to the locus of *arista-less* than the recessive one, such that the genotype of the female parents could be rewritten as $\frac{al\ \ S\ +\ ho}{+\ +\ s\ +}$. In the production of the normal-eyed progeny a crossover was presumed to have occurred at the position of the X: $\frac{al\ \ S}{+\ +} \times \frac{+\ ho}{s\ +}$ to give the $+\ +\ +\ ho$ genotype which was found. The complementary product of such crossing-over would have both alleles in the same chromosome (*al S s* +). Flies arising from eggs of this genotype were evidently not distinguished from the other star-eyed progeny. Since the normal-eyed recombinants were all of one genotype with respect to the marker genes, there was no reason to suppose that the star alleles were associated with a duplicated segment such as was known to occur with Bar. Lewis confirmed that there was no evidence from the salivary gland chromosomes of any structural changes associated with the star alleles.

The occurrence of recombination between the star alleles could be taken to imply that these were mutations of different genes. Lewis (1945) favoured this explanation and went so far as to re-christen 'star-recessive' as 'asteroid'. For reasons to be discussed in Chapter 13, it now appears that this decision was premature.

A discovery in many ways comparable to those of Oliver and of Lewis with *Drosophila* was made by McClintock (1944) when she discovered an anomaly in a series of alleles at the yellow-green-2 locus (*yg*-2) in *Zea mays*. Three mutants which produced yellow-green, pale yellow, and white

seedlings, respectively, were found to be inherited as Mendelian recessives to the normal green plant-colour, and to behave as alleles, with descending dominance in the order quoted. The anomaly was that the heterozygote for the two intermediate members of the series, yellow-green and pale yellow, instead of having yellowish-green leaves as expected, was normal green.

Two alternative explanations of this observation are possible. If the mutants are regarded as alleles, then it is necessary to assume that different parts of the gene are abnormal in the yellow-green and pale yellow mutants, respectively, such that the normal parts can complement each other. Alternatively, it could be argued that these two mutants are not allelic, but are due to mutation of neighbouring genes concerned with a similar function. On this hypothesis, each would carry the dominant normal allele of the other's mutation, and hence the heterozygote would be normal.

The gene (or genes) concerned was known to lie close to one end of a particular chromosome (the short arm of no. 9). From an examination of this chromosome, McClintock found that whereas the green and the yellow-green plants had a normal chromosome, the pale yellow plants had a small terminal segment of the chromosome missing, and the white plants had a slightly larger terminal deficiency. These observations suggested that part of the gene (or one of the two on the two-gene interpretation) was missing in the pale yellow plants, and that the whole gene (or both of them on the two-gene theory) was missing in the white plants.

On either interpretation the bead hypothesis is undermined. On the one-gene theory, the alleles for yellow-green and pale yellow plant colour evidently differ from normal at points which do not correspond, so that in effect the gene has been split and, contrary to expectation, the parts found to show autonomy of action in the production of green leaf-colour in the heterozygote for these alleles. On the two-gene theory, neighbouring genes are presumed to interact in the production of the green colour in this heterozygote. A similar argument can be applied to the *Drosophila* data discussed earlier. There were indications that a comparable situation might apply with the human rhesus blood-group factors. About 85% of white people have been found to possess the rhesus antigen, such that their erythrocytes are agglutinated by the antibodies obtained by immunising rabbits with the blood of the Rhesus monkey *Macacus mulatta*. The remaining 15% are rhesus negative and lack the antigen. Haemolytic disease of the newborn (erythroblastosis foetalis) has been shown to be due to rhesus blood group incompatibility between a rhesus negative mother and a rhesus positive child. The presence of the rhesus antigen was found by Landsteiner and Wiener (1941) to be inherited as a Mendelian dominant. A number of apparently allelic variants within both the rhesus positive (*Rh*) and negative (*rh*) blood groups have been recognised. R. A. Fisher (in Race, 1944) suggested that mutant differences at three closely-linked positions (*C*/*c*, *D*/*d* and *E*/*e*) were each responsible for the presence or absence of a specific antigen, of which *D* and *d* corresponded to rhesus positive and negative, respectively. Further mutant forms at what is possibly a single gene locus have since been recognised (see Race and Sanger, 1962).

It is evident that both the one-gene and the two-gene explanations of the results obtained by Oliver and by Lewis with *Drosophila* and by McClintock with *Zea* represented a breakdown of the classical theory of the gene, and probably likewise with a one gene (*Rh*/*rh*) or a three gene (*C*/*c*, *D*/*d*, *E*/*e*) explanation of the rhesus blood-groups. Were these instances to be regarded as exceptional or typical? This question will be discussed in Chapter 13.

10. *The theory of one gene: one enzyme*

§ 10.1 Introduction

The idea of a connection between genes and enzymes is a long-established one. Garrod (1909) showed that the rare human disease called alkaptonuria appeared to be associated with a failure of the breakdown of the benzene ring in the degradation of phenylalanine and tyrosine, with the result that 2,5-dihydroxyphenylacetic acid is excreted in the urine, instead of the normal fumaric and acetoacetic acids. Bateson and Saunders (1902) had shown, on the basis of data collected by Garrod, that alkaptonuria appeared to be inherited as if due to a single Mendelian recessive gene, and Garrod (1909) pointed out that the splitting of the benzene ring in normal metabolism was likely to be the work of a special enzyme which was presumed to be lacking in congenital alkaptonuria. A few years later this specific enzyme was isolated, and its absence from alkaptonurics confirmed. Bateson (1909, p. 266), in discussing the Mendelian unitary factors, came to the conclusion that 'the consequences of their presence is in so many instances comparable with the effects produced by ferments, that with some confidence we suspect that the operations of some units are in an essential way carried out by the formation of definite substances acting as ferments'.

Indications of how different genes may affect related steps in the development of particular characters in an organism were obtained early in the study of Mendelian heredity. Thus, Bateson, Saunders, and Punnett (1905) crossed two pure-breeding white-flowered varieties of *Lathyrus odoratus* and were surprised to find that the progeny had purple flowers. When these F_1 plants were allowed to self-pollinate, and the F_2 generation grown, they found that out of 651 plants which flowered, 382 had coloured petals and 269 were white. These numbers fit a 9:7 ratio (expected numbers 366·2 and 284·8 respectively, $\chi^2 = 1{\cdot}56$, $n = 1$, $P = 0{\cdot}3$–$0{\cdot}2$). Such a ratio would arise in F_2 if two independent but complementary dominant factors were required for the production of the flower pigment, the 9:7 ratio being a 9:3:3:1 ratio (cf. Chapter 3) in which the last 3 classes were indistinguishable. It was subsequently confirmed by further breeding tests that one parental white-flowered strain had the genotype *CCrr* and the other had the genotype *ccRR*, where *C* and *R* are the complementary dominant genes required for pigment formation.

One possible explanation of these results is that anthocyanin (the pigment in the coloured petals) was formed as the result of two successive biochemical

steps, the end-product of the one forming the substrate of the other. If these steps were controlled by the two genes, respectively, and if the end-product of the first step was colourless, it would be necessary for both reactions to occur, that is, for both *C* and *R* to be present, for the pigment to be formed.

Bateson, Saunders, and Punnett (1905) crossed a pure-breeding pink-flowered strain of *Salvia horminum* with a pure-breeding white-flowered variety. The F_1 plants had purple flowers. On self-pollination or intercrossing these F_1 plants, 255 purple, 92 pink, and 114 white-flowered plants were obtained in F_2. These numbers are in agreement with a 9:3:4 ratio (expected numbers 259·3, 86·4, and 115·3 respectively; $\chi^2 = 0{\cdot}45$, $n = 2$, $P = 0{\cdot}8$), such as would be expected if the last two classes making up a 9:3:3:1 ratio were indistinguishable. Following confirmation of this explanation from further breeding, it was evident that flower colour in *S. horminum* was determined by two independently-inherited Mendelian factors, such that when both dominant genes were present a purple pigment was produced, while one alone (*A*) gave a pink pigment, and the other alone (*B*) the white colour which was also found in the absence of either dominant allele (*aabb*).

A possible explanation in biochemical terms of such a 9:3:4 ratio is that the two genes act sequentially in the synthesis of the purple pigment, gene *A* leading to the production of a pink anthocyanin and gene *B* modifying its molecular structure to give a purple anthocyanin. In the absence of *A*, no anthocyanin is formed and gene *B* is then ineffective, the flower-colour remaining white. Bateson (1907*b*), using the metaphor 'higher and lower', proposed the term *epistatic* for the factor (*A* in this instance) which stands above the other or *hypostatic* factor (*B* in this instance) and determines whether it shall take effect.

Numerous examples of the occurrence in F_2 of modified 9:3:3:1 ratios such as 9:7 and 9:3:4 are known. They point to interaction in development between independently-inherited genes, but the precise nature of such interaction requires identification of the biochemical steps which the genes control. Extensive studies of the nature of such biochemical steps have been made, particularly with the pigments in the fur of mammals, the eyes of insects, and the petals of flowers. Thus, from work on the biochemistry and inheritance of the anthocyanin and anthoxanthin flower pigments, it has been established that individual genes appear to be concerned with specific chemical operations such as methoxylation and glycosidation at particular positions in the molecule (cf. Scott-Moncrieff 1936; Lawrence 1950). This is precisely the kind of result anticipated if there is a relationship between particular genes and specific enzymes.

§ 10.2 Nutritional mutants in fungi and bacteria

A major step forward in the study of biochemical genetics was taken by Beadle and Tatum (1941). They reversed the ordinary procedure, and instead of attempting to work out the chemical basis of known genetic

characters, they set out to determine if and how genes control known biochemical reactions.

Conidia of *Neurospora crassa* and *N. sitophila* were X-rayed, and then used to fertilize a strain of the opposite mating-type. About 2000 of the resulting ascospores were germinated and cultured on a 'complete' medium containing agar, inorganic salts, malt extract, yeast extract and glucose. The ability of the cultures to grow on a 'minimal' medium was then tested. This medium comprised agar (optional), inorganic salts, biotin, and a disaccharide, fat or more complex carbon source. Three mutants were found which grew essentially normally on complete medium and scarcely at all on the minimal medium with sucrose as the carbon source. These strains were then tested systematically by adding particular vitamins, amino-acids, etc. in turn to the minimal medium to determine what substance or substances they were unable to synthesise. In this way it was found that the three mutants had lost the ability to synthesise pyridoxin, thiamin, and para-aminobenzoic acid, respectively. Study of the inheritance of these character-differences showed that each differed from normal by a single gene.

Taking advantage of the method which Beadle and Tatum had devised, Srb and Horowitz (1944) obtained by X- or ultraviolet irradiation 15 mutants of *N. crassa* which lacked the ability to synthesise arginine. When these mutants were crossed with wild-type, it was found that each differed from normal by a single gene. When they were crossed with one another it was found that a number of them gave only arginine-requiring progeny, or in other words appeared to be independent mutations of the same genes. On the other hand, others gave many arginine-independent progeny and were evidently not allelic. When allowance was made for the alleles, the 15 mutants were resolved into mutations of 7 different genes.

These authors also used a second method of testing for allelism based on heterokaryosis (see § 5.4). Beadle and Coonradt (1944) had shown that when two recessive mutants of *Neurospora* of the same mating-type but having different nutritional requirements were grown together on minimal medium, a cell fusion (but no nuclear fusion) occurred to form dikaryotic mycelium, and this heterokaryon had a growth rate similar to that of the normal fungus. In other words, each mutant could supply the other's deficiency. Many combinations were tested, for example, a strain requiring *p*-aminobenzoic acid and another requiring nicotinic acid. Confirmation of heterokaryon formation was obtained by isolating hyphal tips and breeding from them: in this instance, from one hyphal tip it was found that some of the progeny required *p*-aminobenzoic acid and others required nicotinic acid for growth. Beadle and Coonradt also demonstrated the value of such heterokaryons in testing for allelism. Two mutants requiring nicotinic acid for growth were shown to complement one another, or in other words, in the heterokaryon each kind of haploid nucleus could supply what the other lacked. It was concluded that they were due to mutations of different genes. (Later work has shown that this inference is not necessarily valid: see Chapter 13.) Using this method, Srb and Horowitz were able to show

that certain of their arginine mutants were allelic and others not, and they found that this heterokaryon complementation test gave results in agreement with the progeny-testing method.

By testing the ability of the arginine mutants to respond to the related substances ornithine and citrulline, Srb and Horowitz found that those in 4 of the 7 classes (*A–D* in Fig. 10.1(*a*)) would respond to both these substances,

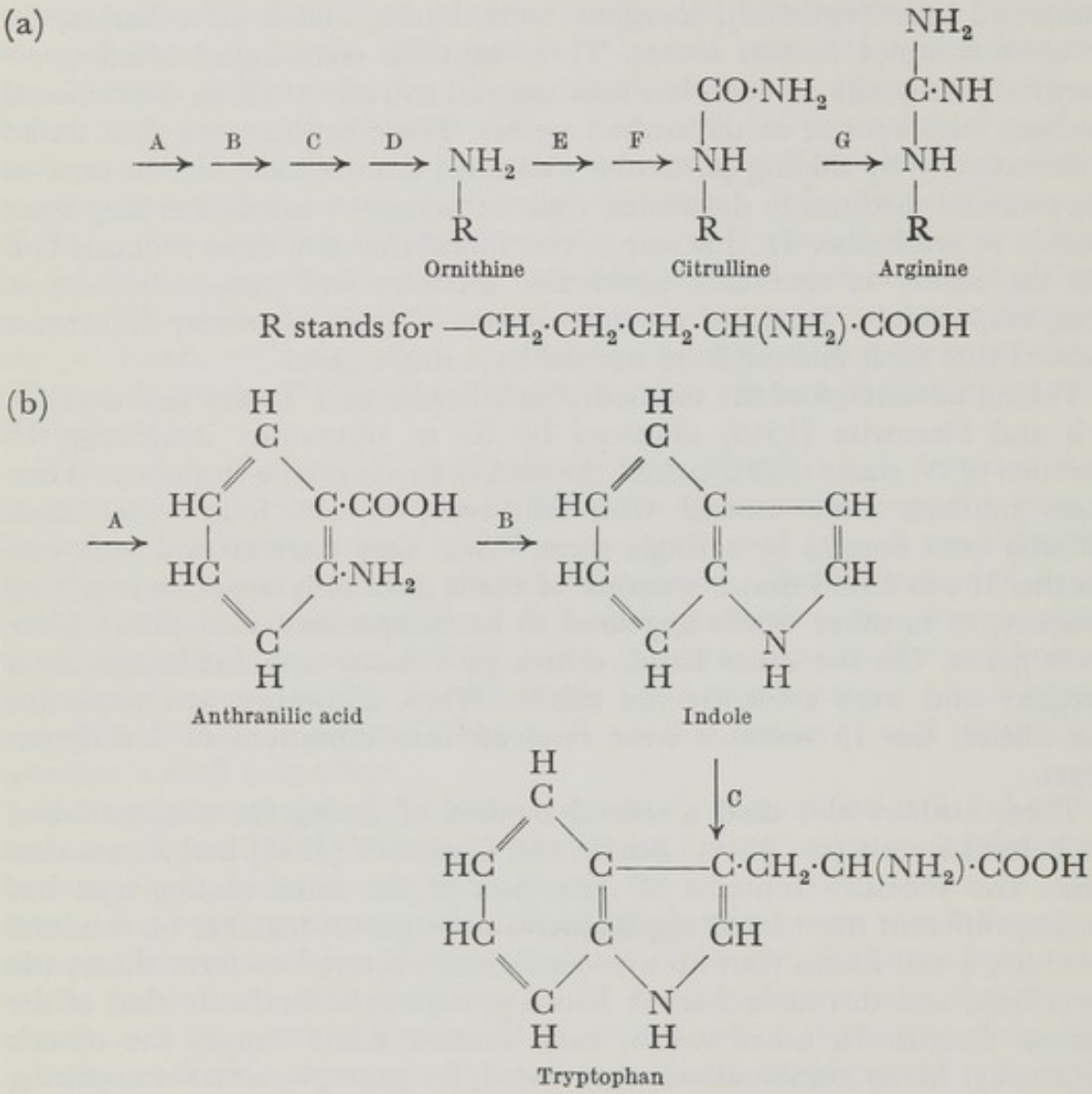


FIGURE 10.1 Steps in the synthesis of (a) arginine and (b) tryptophan in *Neurospora*.

those in a further 2 classes (*E*, *F* in the figure) would respond to ornithine but not to citrulline, and the 7th class (*G* in the figure) would respond to neither substance. They concluded that ornithine and citrulline were precursors, in the order quoted, in the biosynthesis of arginine, and that there was an ordered series of chemical steps, with each gene controlling a single step in a chain of reactions. The mutations were regarded as the loss of the ability to carry out the appropriate step, so that the sequence was blocked at that point. Since there were 7 different genes involved, it was

assumed that there must be at least 7 steps in arginine synthesis. Much more is now known about these steps (cf. Fincham and Day, 1965).

Tatum, Bonner and Beadle (1944) made comparable studies with tryptophan-requiring mutants of *Neurospora*. One (*A* in Fig. 10.1(*b*)) was found able to use either anthranilic acid or indole in place of tryptophan, while another (*B* in the figure), which was non-allelic with the first, grew well on indole but was unable to use anthranilic acid. It thus appeared that anthranilic acid was an intermediary in the synthesis of indole, and thence of tryptophan. Moreover, mutant *B* accumulated anthranilic acid in its tissues, as expected if the succeeding biosynthetic step was blocked. Mitchell and Lein (1948) discovered a third class of tryptophan mutant, *C*, which lacked the ability to couple indole and serine to produce tryptophan, and furthermore, cell-free extracts appeared to lack the appropriate enzyme, tryptophan synthetase, while the *A* and *B* mutants did not. Again, later work along the same lines has provided more information about the steps in tryptophan synthesis (see §13.7).

In view of the remarkable association found in *Neurospora* between mutation of specific genes and blocking of specific biosynthetic steps, it was natural for Beadle (1945) to assume that every biochemical reaction had a specific gene directing its course, and to suppose that the gene's primary and possibly sole function was in directing the configurations of protein molecules.

Critics of the one gene: one enzyme hypothesis pointed out that the method of analysis employed in the *Neurospora* work is a selective one favouring the detection of mutants with single deficiencies, because mutants with multiple defects would probably not survive. This criticism is not easily discounted. Nevertheless, it soon became evident that the apparent one-to-one relation applied very widely, and this tended to weaken though not to invalidate the argument that mutants with multiple effects were being overlooked. The fermentation of a number of sugars by *Saccharomyces cerevisiae* and other yeasts appeared to be controlled by specific genes, and in bacteria mutants were obtained comparable to those found in fungi. Gray and Tatum (1944), by X-ray treatment of cells, obtained a mutant of *Escherichia coli* requiring biotin for growth, and another requiring threonine. This led directly to the discovery by Lederberg and Tatum (1946) of recombination in *E. coli* (see § 16.3), which, in turn, enabled the genetic basis of the biochemical mutants to be established. It soon became evident that, as in fungi, each step in a biosynthetic pathway appeared to be controlled by a specific gene, and in a number of instances it was possible to demonstrate that a specific enzyme was apparently lacking.

Many instances have since been found of mutants associated with the lack of a specific enzyme: Fincham (1959*b*) listed 54 examples in organisms ranging from bacteria to man. A particularly clear example of a relation between a specific gene and a specific enzyme is due to Horowitz, Fling, Macleod and Sueoka (1961), who found 4 different forms of the enzyme tyrosinase in different wild-type strains of *Neurospora crassa*, and the differences were found to be due to allelic mutations.

§ 10.3 Apparent exceptions to the one gene : one enzyme hypothesis

Several apparent exceptions to the one gene-one enzyme hypothesis have been found, but on detailed study they have proved to be in keeping with the hypothesis.

Following irradiation of conidia of *Neurospora crassa* with ultraviolet light, Teas, Horowitz and Fling (1948) discovered a mutant which had a double

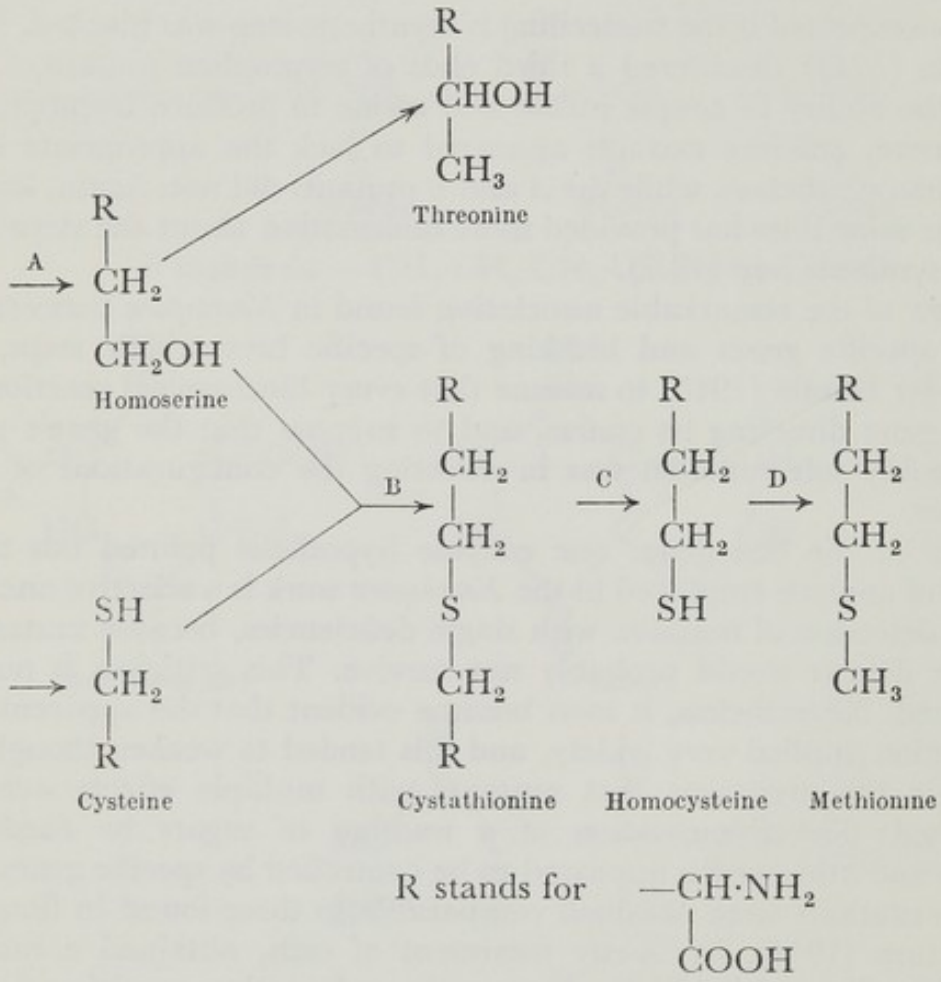
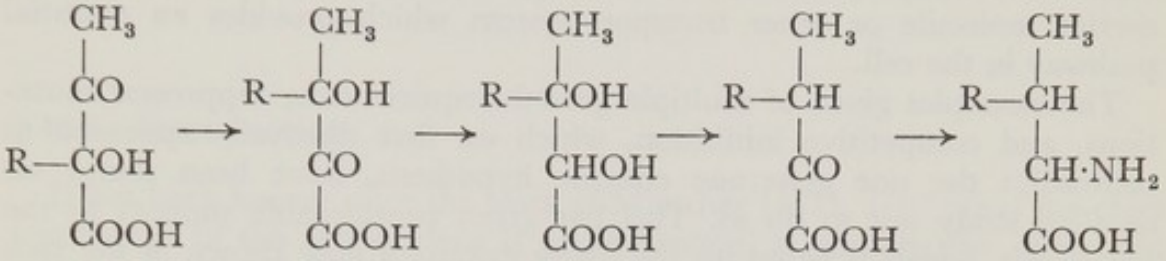


FIGURE 10.2 Steps in the synthesis of threonine and methionine in *Neurospora*.

requirement for growth: it was found to need both methionine and threonine. It was not due to two simultaneous mutations because a cross with wild-type showed that it differed from it by only one gene. Methionine-requiring mutants were already known controlling the steps *B*, *C*, and *D* in Fig. 10.2. Since the methionine requirement of the new mutant was satisfied also by homocysteine or by cystathionine, but not by cysteine, it was inferred that the double requirement was due to the blocking of a biosynthetic step in a common precursor of cystathionine and threonine. This was confirmed when it was discovered that homoserine alone was active in promoting growth. It was concluded that the mutant was blocked in step *A* in Fig. 10.2, and that threonine was derived from homoserine.

Another example of a double requirement was found to have a different explanation. A number of mutants of *N. crassa*, *Escherichia coli* and other organisms are known which require both isoleucine and valine for growth. It appears that the 4 final steps in the synthesis of these amino-acids are essentially the same (see Fig. 10.3), and that the same enzymes control these steps whether isoleucine or valine is being synthesised (see Wagner, Radhakrishnan and Snell, 1958).



R stands for $-CH_3$ in valine pathway
R stands for $-CH_2 \cdot CH_3$ in isoleucine pathway

FIGURE 10.3 The four final steps in the synthesis of valine and isoleucine.

Early in the study of biochemically-deficient mutants in fungi it was discovered that apparent back-mutation to wild-type was sometimes due, not to a reversal of the original mutation, but to mutation of a different gene. The effect of this mutation was to suppress the effect of the original mutation. The suppressor mutation usually showed no linkage to the mutant it suppressed, the two separating from one another in many of the progeny. If the suppressor gene can take over the function of the gene it suppresses, this might imply that two genes can control one enzyme. However, more detailed study, such as has been made of suppressors of tryptophan synthetase deficient mutants (*tryp*-3) in *N. crassa* by Yanofsky and Bonner (1955), has revealed that the suppressor mutants are allele-specific. Thus, one (called su_2) was found to suppress the effect of allele no. 2 at the *tryp*-3 locus, two others (su_3 and su_{24}) suppressed both allele no. 3 and no. 24, while a fourth (su_6) suppressed alleles nos. 2 and 6. The suppressor mutations su_3 and su_{24} appeared to be allelic with one another, but the others were due to mutation at unlinked loci. None was capable of suppressing all the *tryp*-3 alleles: su_2 and su_6 were tested against 25 *tryp*-3 alleles and it was found that only those mentioned above were suppressed.

It was evident that the suppressor genes were not capable of taking over the function of the *tryp*-3 gene. Instead, it seems likely that they act by restoring the functioning of this gene. Such restoration might come about in a number of ways, and there is evidence for diversity of action of different suppressor mutations (see § 14.15 and § 14.17). Irrespective of the precise mechanism of action, which is not known in most instances, it appears that suppressors are to be regarded as modifiers of the functioning of the mutant gene they suppress. Their existence therefore does not conflict with the idea that enzyme structure is essentially determined by one gene.

Another type of complication which has often been encountered in studies of nutritional mutants of fungi is competitive inhibition of growth of, for example, an arginine-requiring mutant by lysine, and *vice versa*. Pontecorvo (1952*a*) found that the growth of heterokaryons of an arginine-requiring and a lysine-requiring mutant of *Aspergillus nidulans* was much affected by the concentrations of these substances in the medium, whereas a diploid strain heterozygous for both mutants was unaffected. It was evident that the site of amino-acid competition was in the cytoplasm, and presumably concerned a carrier molecule or other transport system which provides an essential pathway in the cell.

The examples given of multiple growth-requirements, suppressor mutations, and competitive inhibition, which on first discovery appeared to contradict the one gene:one enzyme hypothesis, have been found on detailed study not to do so. This has given considerable support to the hypothesis, which is based on the many examples now known of the lack of a specific enzyme (or its existence in a modified form) in mutant strains of various organisms. It has now been possible to specify the nature of the relationship between gene and enzyme in much more precise terms, and this is discussed in Chapters 13 and 14.

11. *The DNA theory of the chemical basis of heredity*

§ 11.1 Introduction

It has been known since the work of Miescher (1871, 1897) that the chief constituent of the cell nucleus is nucleoprotein, a combination of nucleic acid and basic proteins. It was later established that the nucleic acid which occurs in combination with protein in the chromosomes is deoxyribonucleic acid (DNA). DNA also occurs in the nuclei of bacteria, where it is associated with little or no protein.

Bacterial geneticists have used the term chromosome for the fine DNA thread found in the nuclei of bacteria and in bacterial viruses. However, these threads differ in a number of respects from the chromosomes of higher organisms. In addition to the chemical difference, the bacterial threads do not show the cycles of behaviour which chromosomes undergo at mitosis and meiosis. Other differences between bacteria and higher organisms in the organisation of their DNA are referred to in later chapters. In order to avoid the confusion caused by this misapplication of the term chromosome to bacteria, the word *chromoneme*, meaning 'coloured thread', has been adopted in the present work for the DNA thread in bacteria and viruses. There is evidence that the primary division of living organisms is not into Plants and Animals, but into the Chromonemal and Chromosomal Kingdoms, since the differences between chromonemes and chromosomes appear to be associated with a number of other fundamental differences in cell structure and organisation (see Appendix 1). In this primary subdivision, the Cyanophyta (Blue-green Algae) belong with the Bacteria. Recognition of these two kingdoms is important, because their differences are so great that conclusions about the mechanism of heredity, based on observations in the one, do not necessarily apply to the other.

For nearly 30 years after the acceptance of the chromosome theory of heredity, it was assumed more or less implicitly that the specificity of the gene resided in the protein part of nucleoprotein. The reason for this assumption was that proteins were known to occur in a very large number of highly specific forms, whereas nucleic acids were not thought to have a structure capable of much variety. DNA was found on hydrolysis to yield the purines, adenine and guanine; the pyrimidines, cytosine and thymine; phosphoric acid; and a sugar, deoxyribose. However, in a period of little more than a decade beginning in the year 1944, a complete revolution of

thought took place, and it is now generally accepted that it is the DNA which forms the chemical basis of heredity.

§ 11.2 Bacterial transformation

The discovery which initiated this remarkable change in ideas was the finding by Avery, MacLeod, and McCarty (1944) that DNA was the carrier of a specific hereditary character in *Diplococcus pneumoniae* (the pneumococcus). Griffith (1928) had discovered the phenomenon of genetic *transformation*. He had taken a virulent strain of the pneumococcus having a polysaccharide capsule surrounding the cell-wall giving the colonies a smooth (*S*) appearance. He heated this strain until all the cells were killed and then mixed with it a small quantity of living cells of an avirulent non-encapsulated strain giving rough (*R*) colonies, and injected the mixture into mice. He found that the mice frequently succumbed to the infection, that living virulent encapsulated (*S*) pneumococci could be isolated from them, and that this character combination of the bacteria was quite stable and was inherited. Control experiments using the living avirulent (*R*) culture alone, and others using the heated virulent (*S*) cells alone, showed no lethal effect of either on the mice, and no production of encapsulated (*S*) pneumococci.

Later, others demonstrated transformation *in vitro*, by growing the avirulent (*R*) cells in a fluid medium containing heat-killed virulent (*S*) cells. Subsequently, cell-free extracts of the *S* strain were obtained which could cause the specific genetic transformation from *R* to *S*. After many years' work, Avery and associates isolated in a highly purified form the substance responsible for this genetic change and showed that it was DNA.

Subsequently, many other characters were shown to be capable of transfer by DNA, both in this and a number of other species of bacteria. In 1952, the importance of DNA in heredity was also demonstrated with bacteriophage.

§ 11.3 Bacteriophage multiplication

Filter-passing agents pathogenic to specific bacterial strains were called bacteriophages before their viral nature, demonstrated by their infectivity and by their dependence for multiplication on their host, was established. Seven different phages, all active on a strain called *B* of *Escherichia coli*, have been given the type numbers 1 to 7 (*T*1, *T*2, etc.). Nos. 3 to 6 were isolated in America from sewage. The sources of the other three do not appear to have been recorded, but were almost certainly similar. These phages can be distinguished by their serological properties, by the range of strains of the host which they will attack, and by the shape and size of the clear area (plaque) which is produced in a bacterial colony by their destruction of the bacterial cells. The *T*-even phages (*T*2, *T*4, *T*6) are similar in many respects, and might be regarded as belonging to the same 'species', but each of the other four evidently belongs to a different species of virus.

The morphology of the *T*-even phages has been revealed in great detail (see Fig. 11.1) from electron micrographs obtained by Brenner, Streisinger, Horne, Champe, Barnett, Benzer, and Rees (1959) using a new technique involving phosphotungstate for negative staining. These viruses are alike in

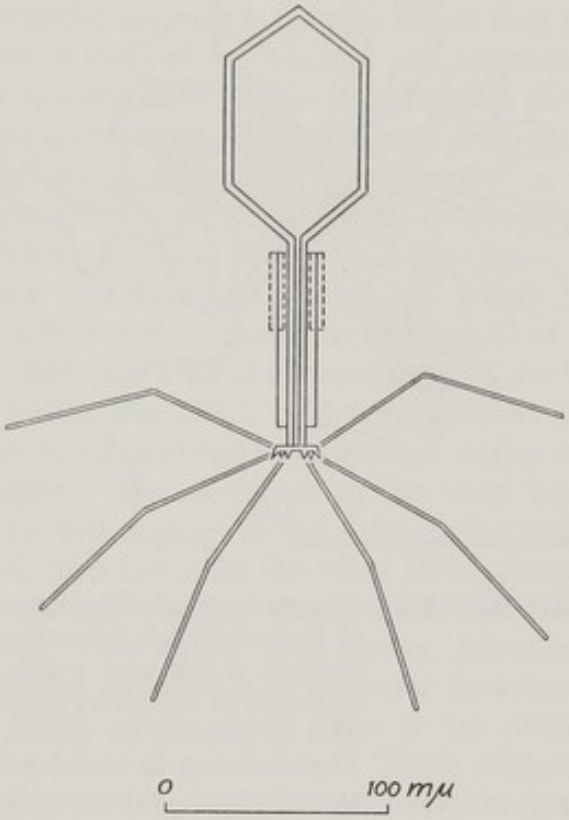


FIGURE 11.1 Diagrammatic representation of the structure of the *T*-even phages of *Escherichia coli*, from the electron micrographs obtained by Brenner *et al.* (1959).

appearance, and are tadpole-shaped with a head and tail. The head is a bipyramidal hexagonal prism measuring about 100 × 65 mμ. The contents are enclosed by a membrane about 3·5 mμ thick. The cylindrical tail, which is about 100 mμ long, consists of a core, surrounded by a contractile sheath. The core, which is attached to the head, is a hollow cylinder of diameter 7 mμ externally and 2·5 mμ internally. The sheath, which is not attached to the head, is about 80 mμ long and 16·5 mμ in diameter when extended. The virus becomes attached to the host cell by the tip of the tail, and the sheath then contracts towards the head to a length of about 35 mμ and diameter 25 mμ externally and 12 mμ internally, exposing the tail end of the core. The sheath appears to be composed of helically arranged subunits. At the tip of the core, where contact with the bacterium is made, electron micrographs have revealed a hexagonal base plate bearing six short spikes and six long tail fibres measuring about 130 × 2 mμ and with a kink in the middle.

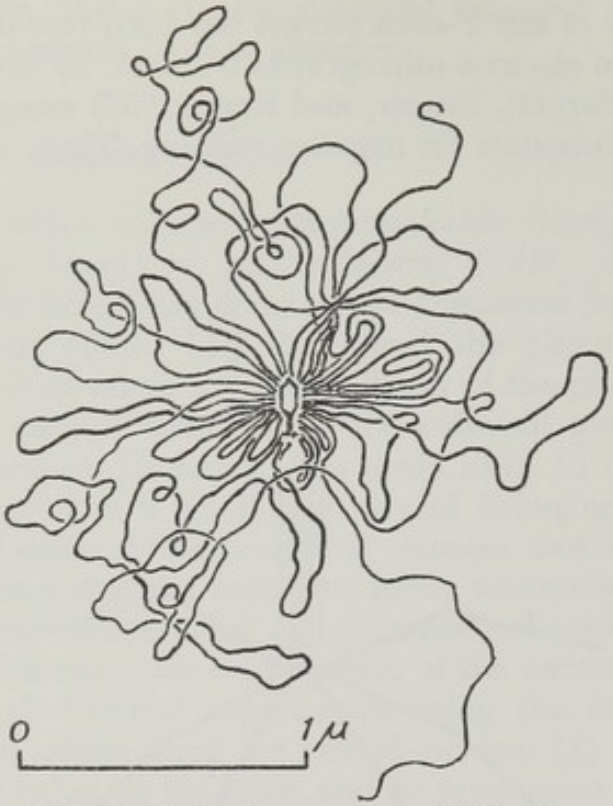


FIGURE 11.2 Appearance of *T*2 phage of *Escherichia coli* following osmotic shock and gentle diffusion of the DNA on a film of protein on water. The drawing is based on an electron micrograph obtained by Kleinschmidt *et al.* (1962).

Physical and chemical studies have established that the *T*-even phages are composed wholly of DNA and protein, and that the DNA, which accounts for 40% by weight of the total, occurs within the head. Brenner *et al.* (1959) have shown that the head membrane, the sheath, and the tail fibres are composed each of a different protein.

If *T*2 phage is suspended in a concentrated solution of sodium chloride and then the solution is rapidly diluted with water, the virus particles suffer an osmotic shock and are inactivated. Under the electron microscope tadpole-shaped ghosts are then seen—empty shells from which the contents have been removed. The shells will specifically adsorb to the bacteria although incapable of producing progeny virus. This adsorption appears to be determined by the tail fibres since detached fibres become attached to bacterial cells with the same specificity as the whole virus. The osmotic shock releases the DNA into the solution. This is shown by the vulnerability of the DNA to the enzyme deoxyribonuclease to which it was previously resistant, no doubt through protection by the protein membrane of the head. This release of DNA has also been beautifully demonstrated by Kleinschmidt *et al.* (1962) who floated the DNA on a film of protein on water and then examined the preparation under the electron microscope (see Fig. 11.2).

Hershey and Chase (1952*b*) isotopically labelled the protein and, in a separate experiment, the DNA of virus *T*2, using radioactive sulphur (^{35}S) for the protein and radioactive phosphorus (^{32}P) for the DNA. After infection of bacteria, unadsorbed virus was removed by centrifugation, and then the bacteria were agitated in a high-speed mixer in order to detach the virus particles from the bacterial cells-walls. It was found that at least 80% of the sulphur had remained at the cell surface and was removed by the agitation, while most of the phosphorus entered the cells. Despite this violent treatment the bacteria remained capable of yielding phage progeny, and it was found that little or none of the sulphur was incorporated in the progeny virus particles, while the phosphorus was transferred to the progeny to the extent of 30% or more. When labelled *T*2 was inactivated by osmotic shock, nearly all the sulphur was found in the ghosts. From these experiments, Hershey and Chase inferred that the bulk, if not all, of the protein of the virus takes no part in infection after the attachment of the virus to the bacterium. On the other hand, the DNA evidently entered the bacterium and played a part in the virus multiplication, since many of the same atoms appeared in the progeny. The structure of the virus bears out these conclusions. The elaborate tail appears to be a device for attaching the virus to the bacterial cell-wall, and injecting the virus DNA through it.

Hershey and Chase's experiments established that the genetic material of the virus is its DNA. This agrees with Avery, MacLeod and McCarty's discovery with *Diplococcus pneumoniae* in showing that hereditary characters may be carried by DNA alone, since little or no protein appears to be associated with the hereditary transmission. These discoveries gave support to the idea that in higher forms of life (chromosomal as distinct from chromonemal organisms) it might also be the DNA alone which was the chemical basis of heredity, with the protein part of the chromosome having some other function.

§ 11.4 The DNA content of nuclei

Boivin, Vendrely, and Vendrely (1948) made chemical analyses of the DNA content of the nuclei of the thymus, liver, pancreas and kidney of *Bos taurus* (Domestic Cattle). The primary method used was hydrolysis, followed by estimation of the deoxyribose content by a specific colour reaction. The number of nuclei in each preparation was estimated, and the DNA content per nucleus calculated. This led to two remarkable discoveries. First, the nuclei of the various tissues were found all to have essentially the same DNA content of approximately 6·5 pg (1 picogram = 10^{-12} g). Secondly, the DNA content of the sperm nuclei was found to be approximately half that of the nuclei of the diploid tissues, namely, 3·4 pg. It appeared that within a species the DNA content of non-dividing nuclei was independent of the tissue or the individual, and dependent solely on the chromosome content (haploid or diploid). Vendrely and Vendrely (1948), in reporting their results in more detail, pointed out that this was a strong argument in favour of the theory that the DNA is the carrier of the hereditary factors.

Mirsky and Ris (1949) obtained similar results for a number of other animal species, and moreover found significant differences in the DNA content of the nuclei of different species: in diploid tissues, the amount of DNA ranged from about 2 pg per nucleus in *Alosa* (Shad) to about 15 pg in *Rana* (Frog). They pointed out that, while supporting the DNA theory of heredity, these results did not necessarily mean that the gene consists of nothing but DNA.

Ris and Mirsky (1949) found that it was possible to obtain reliable information about the relative DNA content of different nuclei by measuring the intensity of pigmentation after staining by the Feulgen reaction. This staining method involves mild hydrolysis in warm dilute hydrochloric acid, followed by staining with decolorised basic fuchsin, and is specific for DNA, since it depends on the Schiff reaction with aldehydes derived from the deoxyribose. These authors showed the existence in the liver of *Rattus* (Rat) of three classes of nuclei with a ratio of intensity of Feulgen staining of 1 : 1·9 : 3·6. This was in agreement with expectation from the knowledge that diploid, tetraploid and octoploid nuclei occur in this tissue.

§ 11.5 The time of replication of DNA

Swift (1950) studied the DNA content of nuclei in developing tissues of *Mus* and *Ambystoma* using the Feulgen staining method. He found DNA values ranging between that of the normal diploid and twice this amount. He concluded that DNA synthesis occurs in interphase. A similar conclusion was reached for plant material by Howard and Pelc (1951*a*) who demonstrated autoradiographically that radioactive phosphorus (^{32}P) was incorporated into nuclei in the meristematic region of *Vicia faba* root-tips during interphase, but not during mitosis. Walker and Yates (1952) confirmed the interphase synthesis by measuring the absorption of ultraviolet light of wavelength 265 mμ by nuclei in living cells in tissue cultures of particular organs of *Gallus*, *Mus*, *Oryctolagus*, *Rana*, and *Triturus*. There is strong absorption by nucleic acids at this wavelength. The previous history of the individual cells had been recorded by phase-contrast photographs, and so the density of each nucleus on the ultraviolet negatives could be plotted against the time since the previous division of the cell. From this it was established that the doubling of the DNA took place in interphase and extended over a comparatively long period of time.

Swift (1950) showed that at meiosis, like mitosis, the replication of DNA took place in interphase before the nuclear division began. By measurement of the intensity of Feulgen staining, he showed that DNA synthesis was completed before prophase of the first division of meiosis in spermatocytes of *Mus*. This pre-meiotic synthesis was confirmed autoradiographically by Taylor (1953) using plant material. Following incorporation of radiophosphorus (^{32}P) into flower-buds of *Lilium longiflorum* and *Tradescantia paludosa*, he found that the period of DNA synthesis in the pollen-mother-cells of *Lilium* ended before the leptotene stage of meiosis, while in *Tradescantia* it extended to early leptotene, but was completed well before the

zygotene stage when homologous chromosomes begin to associate. Confirmation of this time of DNA synthesis in relation to meiosis has been obtained from further autoradiographic studies made by Taylor (1959*b*) with *L. longiflorum*, and by Monesi (1962) with male *Mus musculus* (cf. Pl. 2(*a*)). These observations are important in connection with the mechanism of crossing-over (see Chapter 16).

It appears that the synthesis of the protein of the chromosome also takes place in the interphase before nuclear division. This has been shown by observing autoradiographically the uptake of radioactive sulphur (^{35}S) supplied in sulphate. By this means, Howard and Pelc (1951*b*) demonstrated that protein synthesis at mitosis in *Vicia faba* root-tips occurs at approximately the same time as DNA synthesis, and Taylor and Taylor (1953) showed the same thing for meiosis in flower-buds of *Lilium*. They found there was also some incorporation of ^{35}S into chromosomes whenever the cell was metabolically active as indicated by uptake of ^{35}S into cytoplasmic protein. Taylor (1959*b*) obtained similar results from autoradiographic study of the uptake of glycine labelled with carbon-14 (^{14}C) in the pollen-mother-cells of *L. longiflorum*.

If both DNA and protein synthesis are completed before nuclear division, whether mitotic or meiotic, Darlington's hypothesis (Darlington 1931*b*) that meiosis is to be regarded as a precocious mitosis appears untenable (see § 8.18).

§ 11.6 The base composition of DNA

Almost simultaneously with the discovery that the quantity of DNA per diploid nucleus was a specific character came the discovery that its composition showed a similar specificity. Chargaff, Vischer, Doniger, Green, and Misani (1949) found that in *Bos taurus* the DNA of calf thymus had the same proportion of the four nitrogenous bases adenine, guanine, cytosine, and thymine, as beef spleen, while Vischer, Zamenhof, and Chargaff (1949) found that the DNA of *Saccharomyces cerevisiae* (Yeast) and *Mycobacterium tuberculosis avium* had quite different base compositions from that of *Bos taurus* and from one another. The technique developed for these analyses involved hydrolysis, followed by chromatographic separation of the bases and their quantitative estimation by ultraviolet spectrophotometry.

Chargaff (1950), in reviewing these findings, drew attention to a remarkable feature, namely, that in every instance the number of molecules of the purine adenine liberated in the hydrolysis of the DNA was approximately equal to the number of molecules of the pyrimidine thymine, and likewise the molar quantities of the other purine, guanine, and the other pyrimidine, cytosine, were equal. On the other hand, the proportions of the two pairs varied widely depending on the species. On a molar basis, the adenine-thymine pair formed 30% of the total in *M. tuberculosis*, 58% in *Bos*, 62% in *Homo*, and 64% in *S. cerevisiae*. These proportions were independent of the tissue or individual from which the DNA was obtained, and were evidently characteristic for each species (see Fig. 14.15).

Wyatt (1951) reported that in the DNA of *Bos taurus* about 6% of the cytosine was in fact 5-methylcytosine, and similarly in a number of other animal species the cytosine component included from 1% to 8% of 5-methylcytosine. In *Triticum* sp. (Wheat), he found that no less than 25% of the cytosine component was 5-methylcytosine. In every instance, however, the total molar quantity of cytosine and 5-methylcytosine was equal to the molar quantity of guanine. In the *T*-even viruses of *Escherichia coli*, Wyatt and Cohen (1952) found that the whole of the cytosine of their DNA was replaced by 5-hydroxymethylcytosine, which occurred in approximately equimolar quantities with guanine. On the other hand, the bacterium itself had a normal DNA composition with cytosine and no 5-hydroxymethylcytosine.

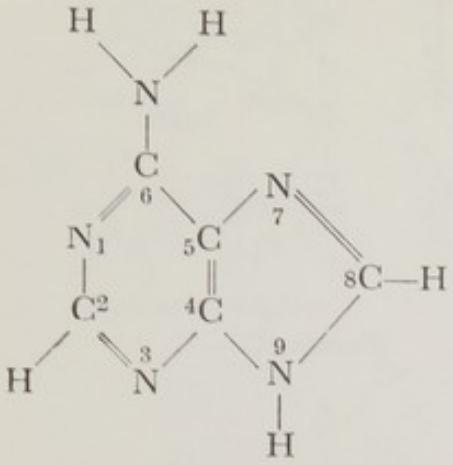
§ 11.7 The structure of DNA

The formulae for the chemical groups which make up DNA, that is, the four primary nitrogenous bases (adenine, guanine, thymine, and cytosine), the sugar (2-deoxy-D-ribose), and phosphoric acid, are given in Fig. 11.3. The glycosidic combination of base and sugar which occurs in DNA is called a *nucleoside*, and the phosphate ester of a nucleoside is known as a *nucleotide*. DNA consists of unbranched polymers of nucleotides. The backbone of these nucleotide chains is quite regular and consists of alternate sugar and phosphate groups joined in 3′,5′-phosphodiester linkages. Side groups consisting of one or other of the four bases are attached one to each sugar molecule. The covalent linkages by which the various groups are united to form the polynucleotide are shown in Fig. 11.4.

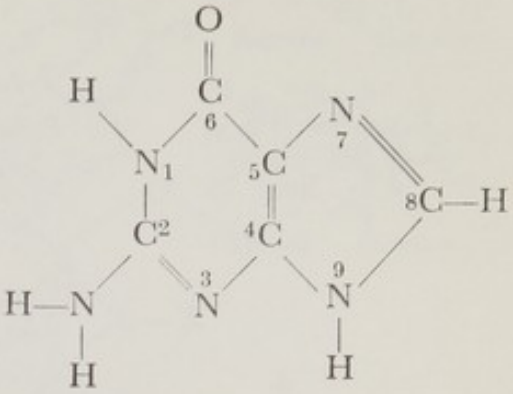
That DNA was composed of such nucleotide chains had been established and the filamentous shape of the molecule was known from electron microscopy, but no satisfactory detailed structure for the molecule as a whole had been proposed, until Watson and Crick (1953*a*) suggested a structure having several remarkable features. They postulated:

(*a*) that the number of polynucleotide chains in the molecule was two,
(*b*) that the chains followed right-handed helices, with 10 bases in each to one turn of the spiral,
(*c*) that the two chains were coiled plectonemically, that is, in an interlocked way, about the same axis,
(*d*) that the sequences of atoms in the two chains ran in opposite directions, or in other words, that one chain was inverted relative to the other,
(*e*) that the phosphates were on the outside of the double helix and the bases on the inside, with their planes set at right angles to the axis of the helix and spaced at intervals of 0·34 mμ along it,
(*f*) that the two chains were held together by hydrogen bonding between the bases, which were joined together in pairs, a single base from one chain being hydrogen-bonded to a single base from the other chain, and
(*g*) that this bonding was of a highly specific kind, such that the purine adenine in either chain was associated with the pyrimidine thymine in the other, and likewise the purine guanine in either chain with the pyrimidine cytosine in the other.

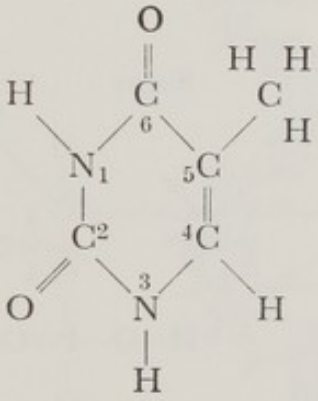
Details of this proposed structure are shown in Fig. 11.5.



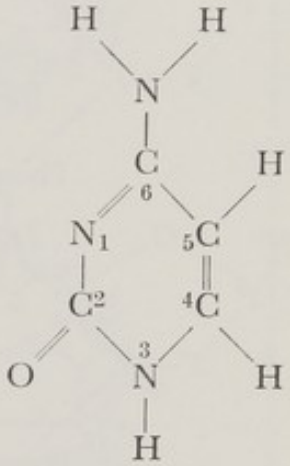
Adenine
(6-aminopurine)



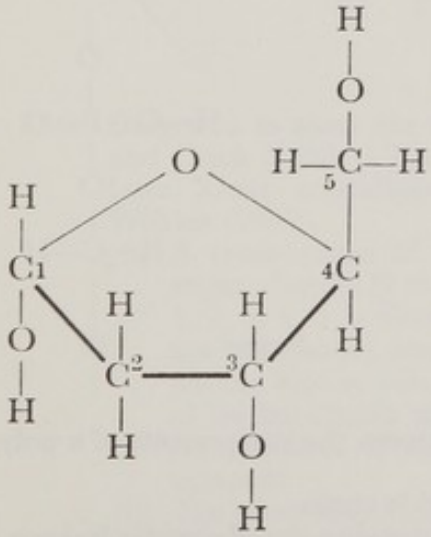
Guanine
(2-amino-6-hydroxypurine)



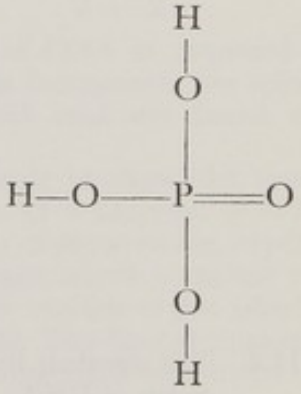
Thymine
(2,6-dihydroxy-5-methylpyrimidine)



Cytosine
(2-hydroxy-6-aminopyrimidine)



2-deoxy-D-ribose



Phosphoric acid

FIGURE 11.3 The components of DNA.

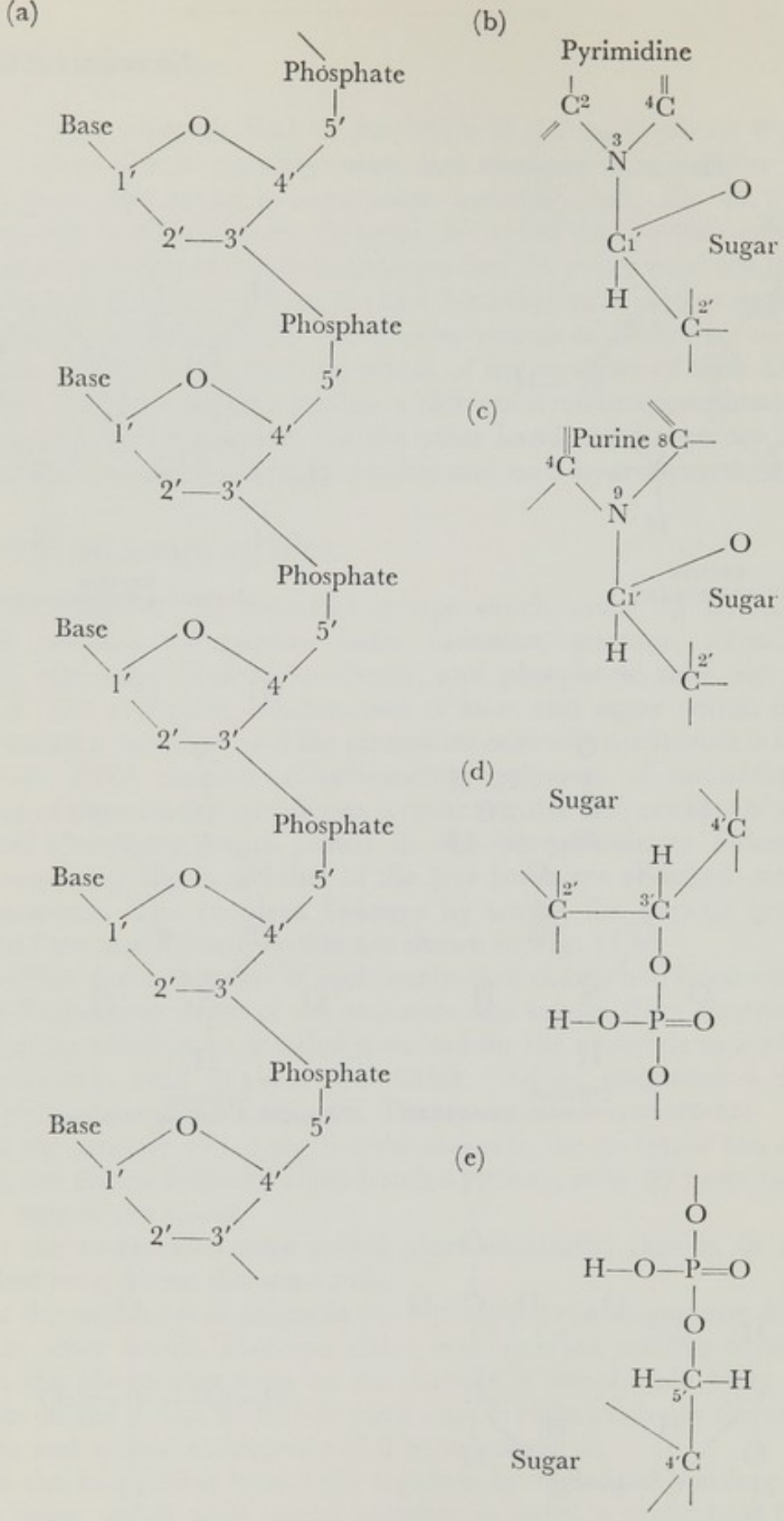


FIGURE 11.4 The covalent linkages between the components of a polynucleotide chain of DNA.

(a) Part of a polynucleotide chain.
(b) Part of a nucleoside showing the glycosidic linkage between a pyrimidine base and the sugar.
(c) As (b) when the base is a purine.
(d) Part of a 3′-nucleotide showing the linkage between nucleoside and phosphoric acid.
(e) As (d) for a 5′-nucleotide.

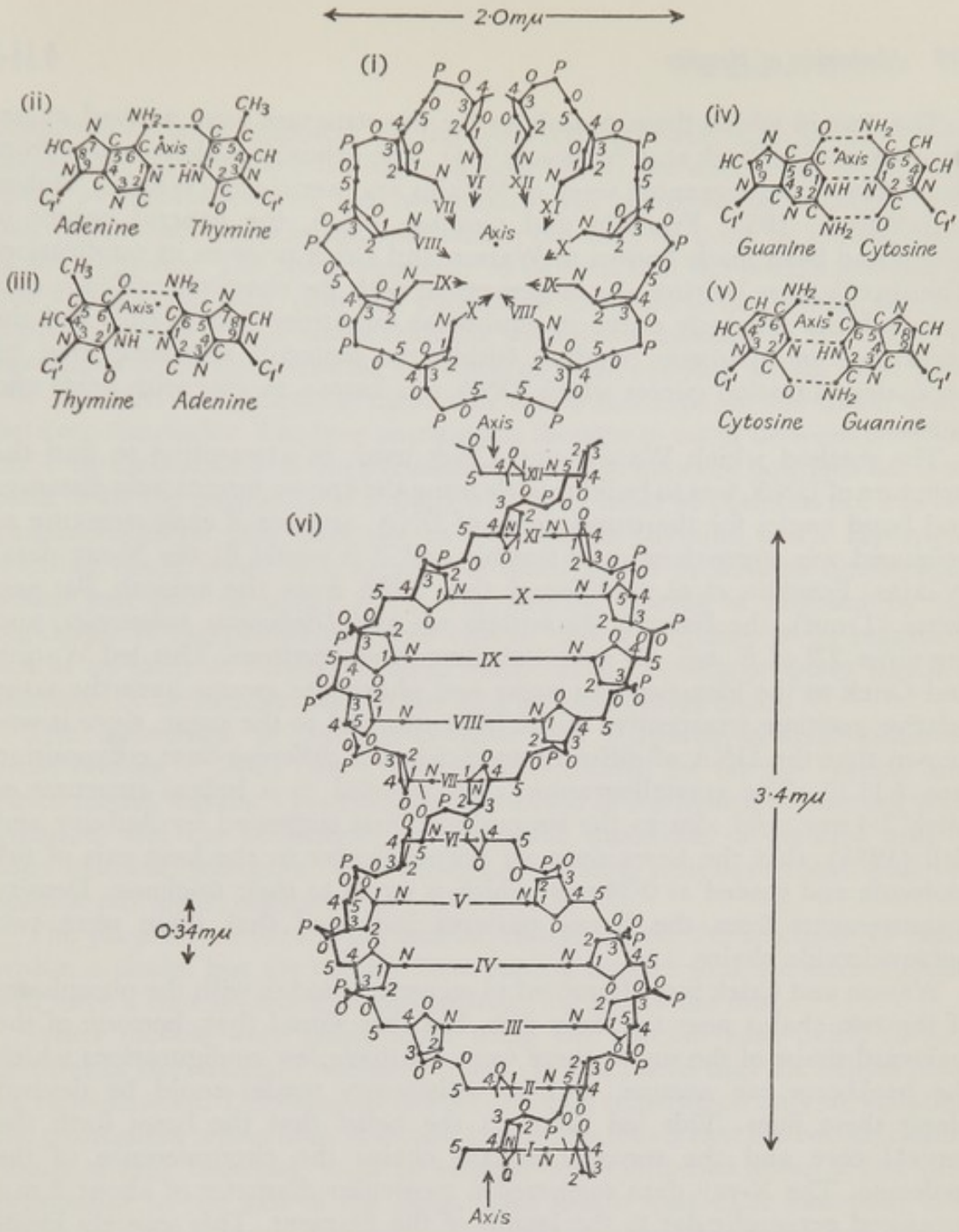


FIGURE 11.5 Diagrams to show the structure of DNA as proposed by Watson and Crick (1953*a*). The diagrams incorporate the inferences from later X-ray crystallographic work and are based on those of Wilkins (1957).

(i) A cross-section of the molecule to show the two phosphate sugar chains. N = Nitrogen. O = Oxygen. P = Phosphorus. 1, 2, 3, 4, 5 = Carbon atoms of deoxyribose. Hydrogen atoms are not shown, nor the oxygen atoms attached to the phosphorus atoms, other than the oxygen atoms which form part of the backbones of the chains. The Roman numerals indicate the positions of successive base pairs numbered from below upwards.

(ii) The adenine-thymine base pair.

(iii) The thymine-adenine base pair.

(iv) The guanine-cytosine base pair.

(v) The cytosine-guanine base pair.

(vi) The molecule seen from the side, lettered and numbered as in (i). The phosphate sugar chains in the foreground are thickened.

The way in which these postulates for the structure were arrived at has been set out by Crick and Watson (1954). The primary evidence came from the X-ray crystallographic work of Wilkins and associates (Wilkins, Stokes, and Wilson, 1953; Franklin and Gosling, 1953), the general nature of which had been made known to Watson and Crick prior to its publication. The specific base pairing was in agreement with the chemical evidence that certain bases regularly occur in equimolar quantities (see § 11.6), and the occurrence of hydrogen bonding within the molecule was supported by anomalous titration curves which DNA was known to give with acids and bases.

The method which Watson and Crick used, in attempting to find the structure of DNA, was to build models using the known interatomic distances and bond angles for the constituents of DNA, and see if each structure so produced was stereochemically feasible and if it would fit the X-ray data. Wilkins, Franklin *et al.* had found that DNA from the animals *Bos* and *Salmo* (Trout), the bacteria *Escherichia coli* and *Diplococcus pneumoniae*, and the virus *T*2 of *E. coli*, all gave the same X-ray pattern. This led Watson and Crick to the idea that the sugar and phosphate groups have the same relative positions irrespective of the base attached to the sugar, since it was known that the DNA of different species had a different base composition (see § 11.6). The crystallographic data pointed to a helical structure of pitch 3·4 mμ, and also to the supposition, first suggested by Astbury and Bell (1938), that the bases were set perpendicular to the long axis of the molecule and spaced at 0·34 mμ, which is equal to their thickness. Density measurements from the X-ray patterns indicated that there were two polynucleotide chains.

Watson and Crick first attempted to construct models with the phosphates of the two chains near the helix axis, but they found that, because of the awkward shape of the sugar, there were relatively few configurations which the backbone can assume, and no satisfactory model could be devised along these lines. This led them to the belief that the bases form the central core and the sugar-phosphate chains the circumference of the molecule. The X-ray data suggested a molecular diameter of about 2 mμ measured perpendicular to the length of the filament. This severely limits the types of model that can be constructed, and it appeared probable that each chain was nearly fully extended and made one revolution of the helix every 3·4 mμ. The two chains would have to be intertwined because it is impossible to have paranemic coiling (a pair of helices which can be directly separated laterally) with two regular simple helices going round the same axis.

Watson and Crick considered it likely that the two chains would be held together by hydrogen bonds between the bases. These bonds are strongly directional in character, and can form only in the plane of the bases. However, since within one chain the bases appeared to be set partially on top of each other like a staggered pile of plates, hydrogen bonds can be expected to form only between bases belonging to different chains, thereby uniting the bases in pairs. If each sugar-phosphate chain is in the form of a regular

helix, as had been supposed, the link between sugar and base (the glycosidic bond) will always have the same orientation to the axis of the helix. The space available between the two chains will therefore be constant, and pairing between bases in opposite chains can only be done by joining bases of the right size. The space appeared to be such that the purine adenine in one chain could pair with the pyrimidine thymine in the other, and the purine guanine in one chain with the pyrimidine cytosine in the other, in agreement with the chemical evidence of their equality within each pair. For all other combinations there was either too little or too much space between the chains. The base pairing was thought to occur between positions no. 1 on the purine and the pyrimidine, and also between positions no. 6 on each, with the possibility of a third hydrogen bond at positions no. 2 in the guanine-cytosine pair (but not in the adenine-thymine pair). Hydrogen bonding could occur at a number of other places in isolated nucleotides, which can pair in many ways. The specific pairing is imposed by the regularity of the phosphate-sugar chains, and is therefore to be regarded, not as an intrinsic feature of these nucleotides, but as a property which is manifest by them when they occur in a polynucleotide chain.

The occurrence of 5-methylcytosine and 5-hydroxymethylcytosine in some sources of DNA (see § 11.6) is unaccounted for on the Watson and Crick model, but the 5 position is not involved in the hydrogen bonding with guanine, and the occurrence of equimolar quantities of guanine and of total cytosine, whichever derivatives are present, is in agreement with the specific pairing hypothesis.

The proposed structure imposes no restriction on the sequence of bases within a chain, but the specific base-pairing implies that the second chain will have a base sequence complementary to that of the first.

When models were constructed using the specific base pairs, further features of the structure became evident. It was found that the helices must be right-handed. Left-handed helices could be constructed only by violating the permissible separations of atoms. It was also found that the two glycosidic bonds of each base-pair must be symmetrically disposed about a line perpendicular to the axis of the helix. This means that each base-pair, if it could be detached and inverted, would fit between the chains just as well the other way round (see Fig. 11.5(ii)–(v)). It follows that all four bases may occur in both chains. Furthermore, one nucleotide chain must always be inverted relative to the other (but with the complementary base sequence). The sequence of atoms in the backbone of one chain will be

$$\ldots\text{—}C_3\text{—}C_4\text{—}C_5\text{—}O\text{—}P\text{—}O\text{—}C_3\text{—}C_4\text{—}C_5\text{—}\ldots$$

and in the same physical direction in the other it will be

$$\ldots\text{—}C_5\text{—}C_4\text{—}C_3\text{—}O\text{—}P\text{—}O\text{—}C_5\text{—}C_4\text{—}C_3\text{—}\ldots$$

(see Fig. 11.5(vi)).

The Watson and Crick model obtained immediate support from the work of Wilkins, Stokes, and Wilson (1953) and of Franklin and Gosling (1953), who, independently of Watson and Crick, deduced that the structure was

probably helical, that the phosphates were on the outside, and that there were two unequally spaced co-axial components. Nevertheless, some features remained hypothetical until the model had been tested with experimental X-ray evidence. This was obtained by Wilkins and associates, when Feughelman, Langridge, Seeds, Stokes, Wilson, Hooper, Wilkins, Barclay, and Hamilton (1955) showed that the Watson and Crick structure was in all essentials correct, although some modification of details was necessary, notably the placing of the bases closer to the axis of the helix. It is this slightly modified version which is shown in Fig. 11.5.

§ 11.8 The biological implications of the structure of DNA

Watson and Crick (1953*b*, 1954) had drawn attention to the genetical implications of their proposed structure for DNA. That this structure was established as substantially correct did not necessarily imply that the biological hypotheses were also true. These hypotheses were:

1. that replication occurs by breakage of the hydrogen bonds, unwinding and separation of the two chains, and the formation of a new complementary chain alongside each, and
2. that the precise sequence of the bases in the nucleotide chain is the code which carries the genetical information.

The first of these hypotheses is discussed in Chapter 12, and the second in Chapters 13 and 14. As a corollary to the sequence hypothesis, Watson and Crick suggested that mutation may be due to a base occasionally occurring in one of its less likely tautomeric forms, so that at replication the wrong base is inserted at this position in the complementary chain. The molecular basis of mutation is discussed in § 14.2. Watson and Crick also suggested that pairing between homologous chromosomes at meiosis may depend on pairing between specific bases.

The brilliant idea underlying the Watson and Crick model of DNA was that there were two complementary nucleotide chains, and that replication of the gene involved each chain acting as a template or mould for the formation of its complement. Such a mechanism for copying the gene would not require gene specificity to be transferred to protein and then back to DNA again, such as previous hypotheses of self-duplication had assumed to be necessary. Watson and Crick's idea thus gave support to the theory that the chemical basis of heredity might be DNA alone. This theory was based on Avery, MacLeod, and McCarty's discovery that DNA alone, without protein, appeared to be the carrier of hereditary factors in *Diplococcus pneumoniae* (see § 11.2), and Hershey and Chase's comparable discovery for virus *T*2 of *Escherichia coli* (see § 11.3). Applied to higher forms of life, this would mean that the protein part of the nucleoprotein of the chromosome played no direct part in heredity. This idea was diametrically opposed to that which had been generally held prior to 1944.

12. *The theory of semi-conservative replication of DNA*

§ 12.1 Introduction

Delbrück and Stent (1957) gave the name semi-conservative to the mechanism of replication of DNA postulated by Watson and Crick (1953*b*), since each new DNA molecule would consist of one old nucleotide chain and one new one, complementary to the old. Watson and Crick (1953*b*, 1954) had visualised the replication process as involving breakage of the hydrogen bonds between the two chains, followed by uncoiling, and synthesis of a new complementary chain alongside each old one. They assumed that the uncoiling and separation of the two chains would occur progressively from one end of the molecule like the opening of a zip-fastener, and that the new synthesis would follow at once, and hence also occur progressively along the molecule. This mechanism would imply that the synthesis would extend from the same physical end in both chains. Owing to the inverted polarity of the two chains, this would mean that in a chemical sense the synthesis was occurring in opposite directions in the two chains. Fig. 12.1 is a diagrammatic representation of this replication process.

§ 12.2 Evidence for semi-conservative replication of chromosomes

One of the first indications that the replication of DNA might be occurring in the way that Watson and Crick had suggested was obtained by Taylor, Woods and Hughes (1957) with root-tips of *Vicia faba* (Broad Bean) labelled with tritiated thymidine. Thymidine is the nucleoside of thymine and deoxyribose and is incorporated exclusively into DNA. Tritium (^{3}H) has the great advantage over other radioactive isotopes that the β-particles which it emits are of such low energy that they will penetrate only for a distance of about 1 μ in photographic emulsion. This means that the position of the labelled atoms can be determined with corresponding precision.

Vicia faba was chosen because it has large chromosomes and because Howard and Pelc (1951*a*) had established the time of replication of the DNA in the meristematic cells of the root-tip in relation to mitosis. At room temperature mitoses succeed one another at about 24-hour intervals, but the divisions are not synchronised so that at any given time there are nuclei at all stages of the mitotic cycle. The DNA replication extends over an 8-hour period ending about 8 hours before metaphase. Accordingly, if *Vicia faba*

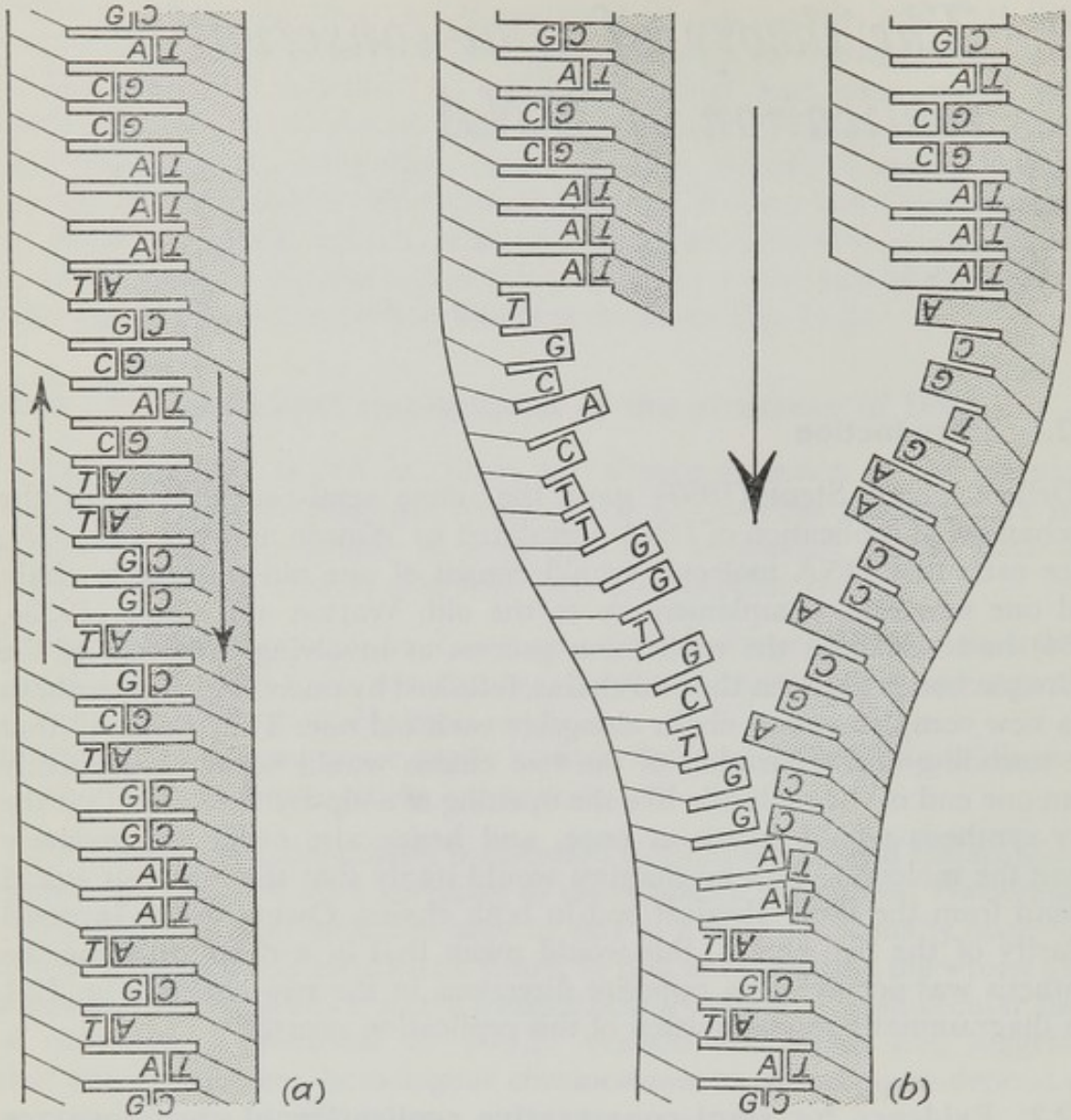


FIGURE 12.1 Diagrammatic representation of the method of replication of DNA proposed by Watson and Crick.
(*a*) Part of a DNA molecule. The arrows indicate the polarity of the two nucleotide-chains. Their spiral coiling is not shown. *A* = adenine, *C* = cytosine, *G* = guanine, *T* = thymine.
(*b*) The molecule in process of replication. The arrow shows the direction in which chain separation and synthesis occur.

seedlings are grown in tritiated thymidine solution for 8 hours, then thoroughly washed with water, and some 8 hours later the root-tips are removed, fixed, stained by the Feulgen method, and squashed on microscope slides, then any cells at metaphase should have incorporated tritiated thymidine at the preceding DNA replication. Photographic film is then placed in contact with the squashed cells, and the preparation is kept in the dark for about two weeks before developing the film to find the position of the tritium.

The technique which Taylor, Woods and Hughes adopted differed slightly from this. During the interval between washing to remove the

tritiated thymidine and fixing, the roots were placed in a non-radioactive mineral solution containing colchicine. There were two reasons for introducing colchicine. In the first place, colchicine causes the two chromatids of each chromosome to diverge, being held together only at the centromere. This facilitates observation of the presence or absence of the isotope in the individual chromatids. Secondly, colchicine inhibits the formation of the mitotic spindle, with the result that the chromosome number is doubled at each mitosis. Hence, the number of mitoses which have occurred since the roots entered the colchicine can be determined from the chromosome number. Taylor *et al.* kept some of the roots in the colchicine solution for 10 hours before fixing, and others for 34 hours.

Cells at metaphase after 10 hours in colchicine had the normal diploid chromosome number of 12, and the two chromatids of each chromosome were found to contain tritium, indicated by dark grains of silver above each chromatid where the photographic emulsion had been exposed. Each chromatid was uniformly labelled, and the two chromatids of a pair equally. Many of the cells at metaphase after 34 hours in colchicine contained 24 chromosomes, as was to be expected if a second mitotic cycle had intervened before the fixation. These 24-chromosome cells showed a remarkable feature: of the two chromatids making up each chromosome, one contained tritium and the other did not. A few of the metaphases after 34 hours in colchicine showed 48 chromosomes, indicating that two successive mitoses had occurred in the colchicine and implying that the 24-hour division cycle was by no means constant. In the 48-chromosome cells, half the chromosomes in each cell showed one labelled and one unlabelled chromatid, while the other half showed no label in either chromatid. The washing prior to placing the roots in the colchicine solution was evidently sufficient to remove the isotope solution, so that subsequent replications of the DNA took place in the absence of labelled thymidine.

These remarkable observations are shown diagrammatically in Fig. 12.2(*a*). It is evident that as far as its DNA is concerned, the chromosome is composed of two sub-units, one old and one new, which extend the whole length of the chromosome, and are such that the DNA of each sub-unit is conserved. This inference is shown in Fig. 12.2(*b*). The chromosome evidently replicates semi-conservatively, but it does not follow from this that the DNA molecule or molecules that form part of it necessarily also show this method of replication. The results are compatible with each of the following structures:

(*a*) The chromosome contains a single giant DNA molecule which replicates semi-conservatively in the way Watson and Crick proposed.

(*b*) The chromosome contains a number of DNA molecules each replicating semi-conservatively and held together in such a way that the new nucleotide chains form a unit which at the next replication separates from the unit formed by the old chains.

(*c*) The chromosome sub-units contain one or a number of DNA molecules replicating conservatively, that is, maintaining the whole molecular structure through the replication process, and the new molecule or

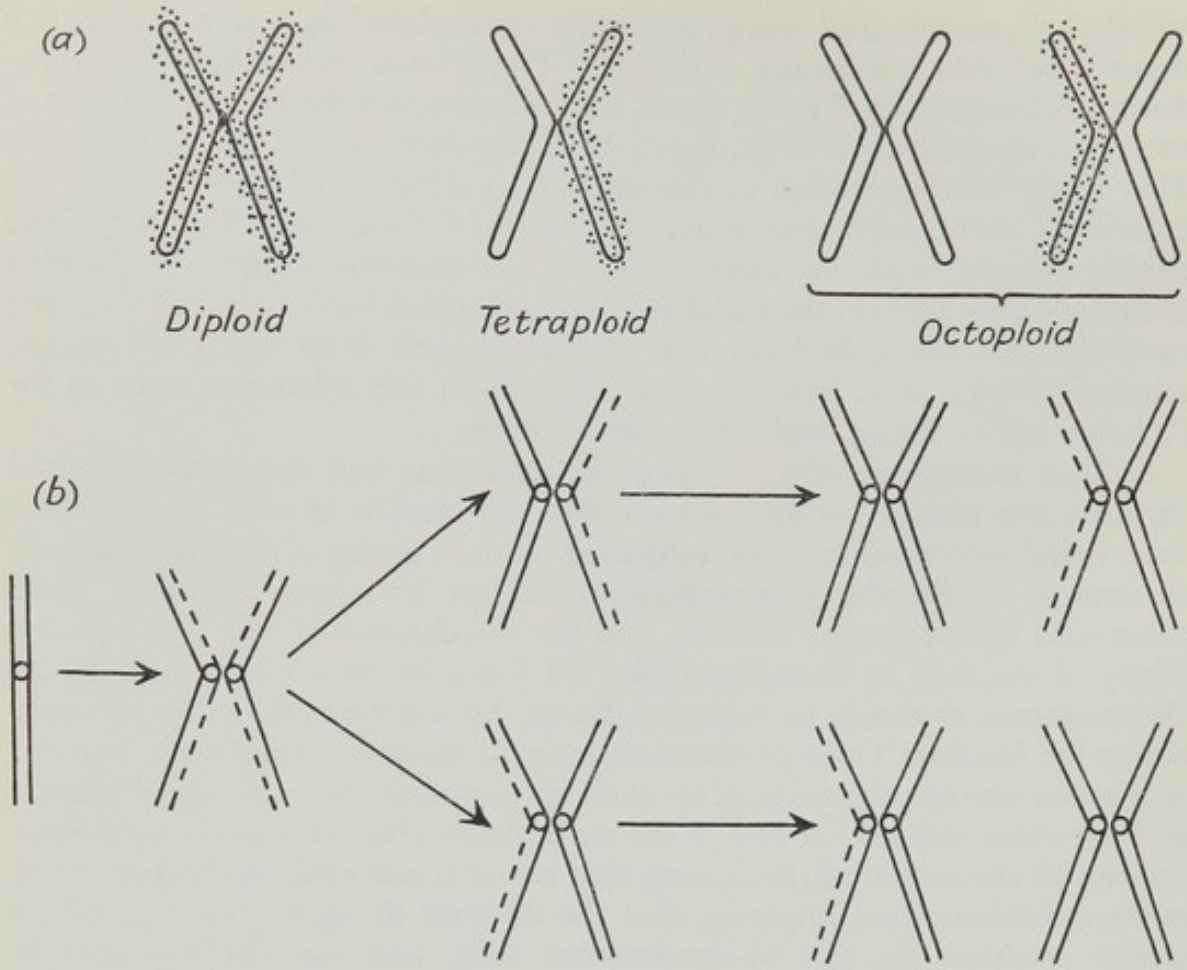


FIGURE 12.2 Diagram to show the observations and inferences made by Taylor *et al.* (1957) from autoradiographs of the chromosomes of *Vicia faba* in root-tips grown in tritiated thymidine for 8 hours.

(*a*) Observations. The drawings represent chromosomes at metaphase of mitosis. Following colchicine treatment, the two chromatids of each chromosome are held together only at the centromere. The dots represent silver grains in the photographic emulsion and indicate the position of the tritium incorporated into DNA.

(*b*) Inferences. The lines represent the two longitudinally-arranged sub-units inferred to exist within each chromatid. Broken lines indicate sub-units containing tritium, and unbroken lines sub-units without tritium. Circles represent centromeres. Of each group of 4 lines representing a colchicine-metaphase chromosome, the two outer lines represent old and the two inner new sub-units.

molecules form a unit which at the next replication separates from the unit formed by the old molecule or molecules.

Although their results are thus open to a variety of interpretations, a further observation led to more information about the nature of the chromosomal sub-units, and made it appear highly probable that the DNA molecules were replicating semi-conservatively. Taylor *et al.* noticed that in some of the 24-chromosome cells the tritium was in one chromatid for part of the length of the chromosome, and in the other chromatid for the remainder of the length. It appeared that an exchange of segments had occurred between the two chromatids.

Taylor (1958) investigated these exchanges in more detail, using *Bellevalia romana* as his experimental material, since it has only 8 chromosomes in the diploid cells, and two of the 4 pairs of chromosomes are individually recognisable. Using essentially the same procedure as before but rather different timing (6–8 hours in the isotope, then 10 hours in a mineral solution free of isotope, and then 12–14 hours in colchicine solution before fixation), Taylor found that exchanges between chromatids were quite frequent, and moreover within one cell two homologous chromosomes often showed exchanges between their chromatids at corresponding positions. These twin exchanges, as he called them, evidently reflected a single exchange before the chromosomes had separated. Observation of the 4 individuals of chromosome no. 1 (the largest chromosome) in each of 18 tetraploid cells showed 15 single exchanges and 36 twin exchanges (72 apparent exchanges). For the other chromosomes the frequencies were comparable but lower, as expected if the exchange frequency were proportional to chromosome length. All the exchanges observed appeared to involve both sub-units of each chromatid.

Let the frequency of exchange for a particular chromosome be denoted by p for the replication in the presence of tritiated thymidine and by q for the subsequent replication in the absence of the label. Exchanges could presumably occur at any time during the interval between replication and the prophase of the mitosis which follows it. If the two sub-units composing each chromatid are alike, such that rejoining is at random, the expected frequency of single exchanges observed at the tetraploid stage is $\frac{1}{2}p + 2q$ and of twin exchanges is $\frac{1}{4}p$ (see Fig. 12.3). The observed frequencies conflict with this hypothesis, since with $\frac{1}{2}p + 2q = \frac{15}{36} = 0{\cdot}42$ and $\frac{1}{4}p = \frac{36}{36} = 1$, q must be negative. On the other hand, if the two sub-units of each chromatid have a structural difference and reunion is restricted to within like sub-units, the expected frequencies of single and twin exchanges are $2q$ and p, respectively (see Fig. 12.4). The observed data give

$$p = \frac{36}{36} = 1 \qquad \text{and} \qquad q = \frac{7{\cdot}5}{36} = 0{\cdot}21.$$

The lower frequency (q) of exchanges at the mitosis in the absence of tritiated thymidine might be due to the lower tritium concentration or to the presence of colchicine, which is known to inhibit X-ray-induced chromosomal aberrations.

Taylor (1959*a*) found that $p = q$ if colchicine is present throughout the experiment, the roots being placed in colchicine from two hours before the beginning of the tritiated thymidine treatment. In 24 tetraploid cells following such treatment, chromosome 1 showed 26 single exchanges and 14 twins, giving

$$p = \frac{14}{48} = 0{\cdot}29 \qquad \text{and} \qquad q = \frac{13}{48} = 0{\cdot}27.$$

The conclusion reached from the relative frequency of single and twin exchanges was that the sub-units of the chromosome are structurally different, such that reunion can occur only between similar ones. This inference,

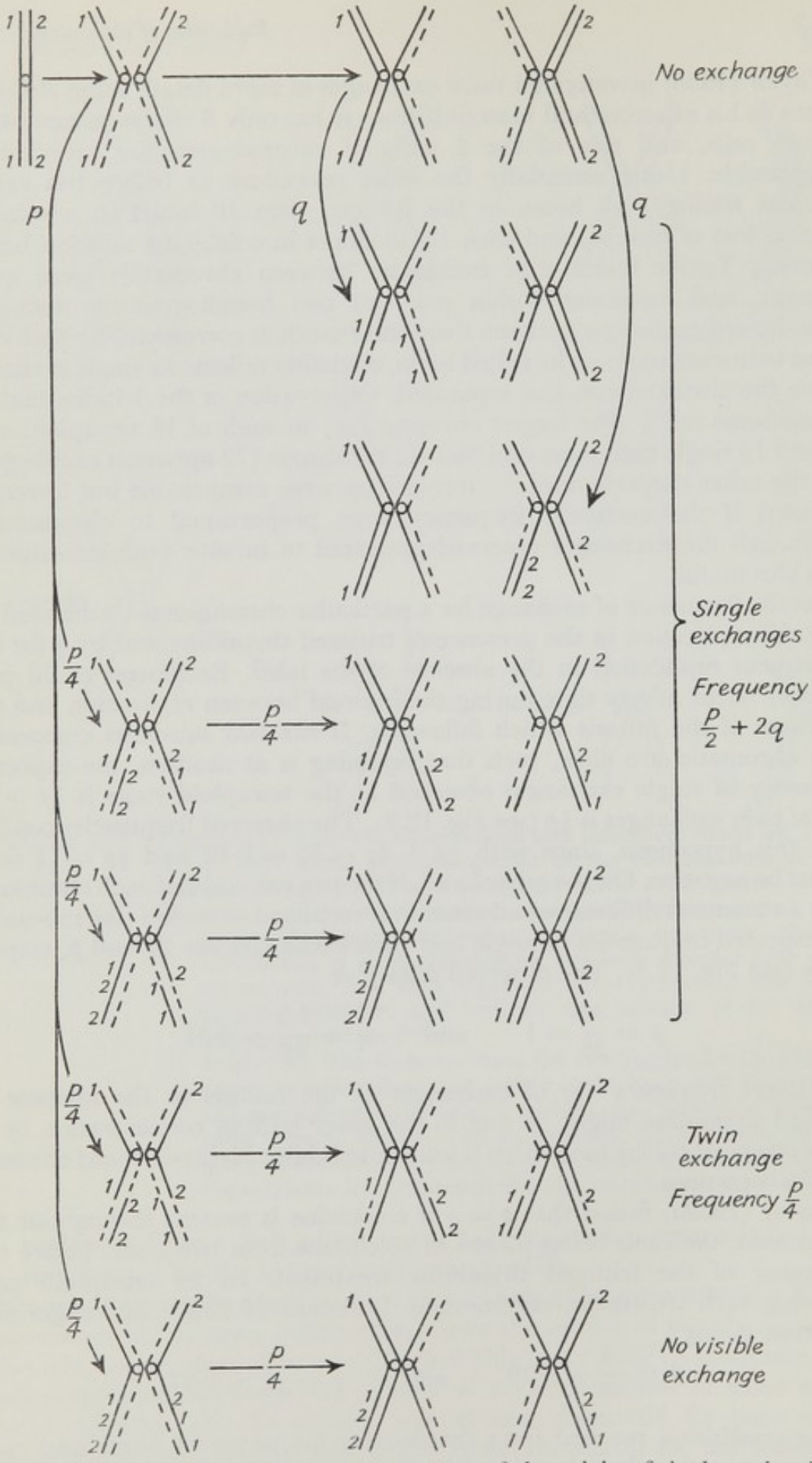


FIGURE 12.3 Diagrammatic representation of the origin of single and twin exchanges between sister-chromatids, as revealed by tritium labelling, if rejoining can occur freely between the broken ends of sub-units. The lines and circles have the same meaning as in Fig. 12.2(*b*). The numbers 1 and 2 show the fate of the segments of the two original sub-units.

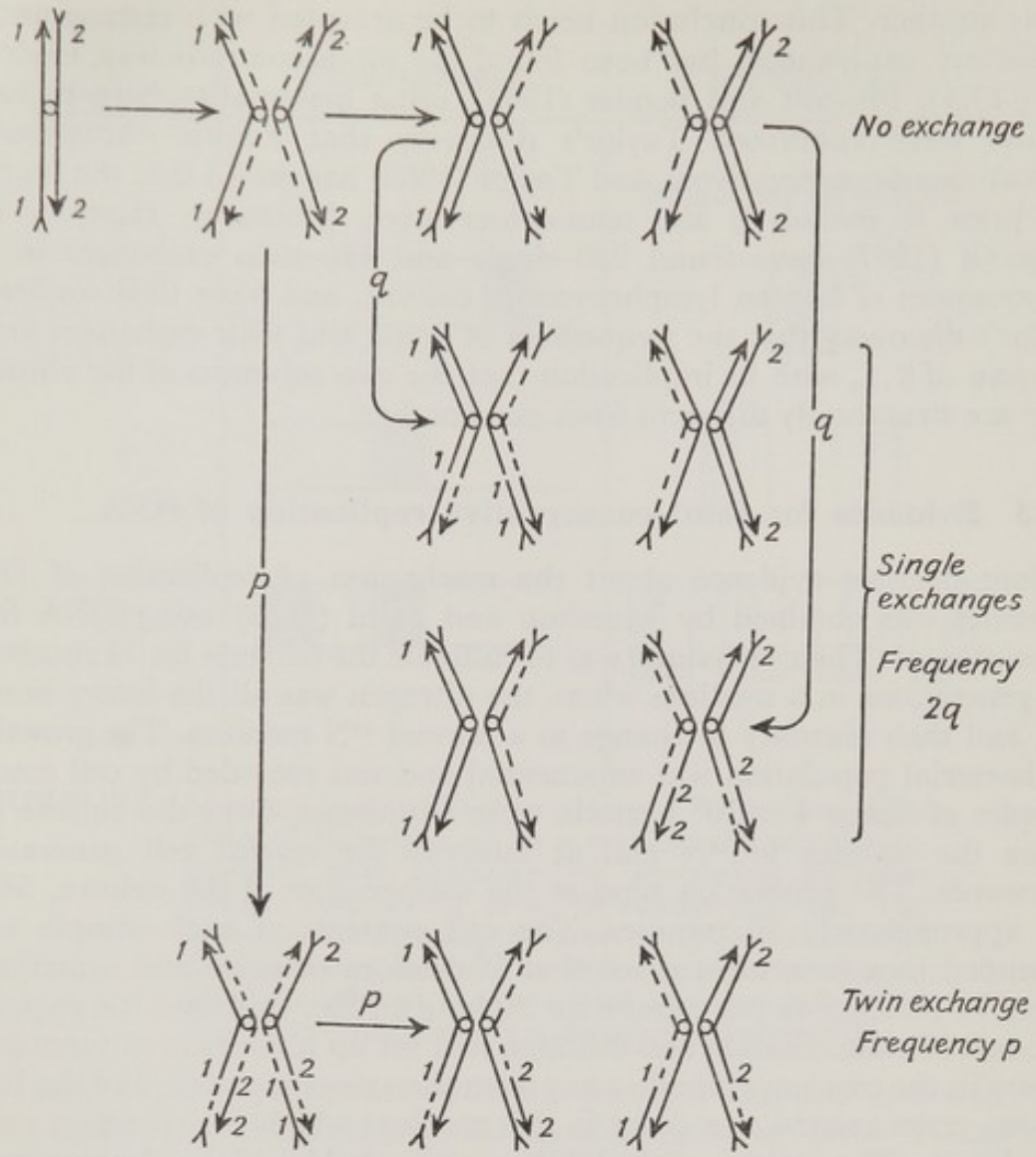


FIGURE 12.4 Diagrammatic representation of the origin of single and twin exchanges between sister-chromatids, as revealed by tritium labelling, if the two sub-units of each chromatid are structurally different, and if rejoining between the broken ends of sub-units can occur only between similar sub-units. The lines, circles and numbers have the same meaning as in Fig. 12.3. The structural difference between the sub-units is indicated by arrows.

taken in conjunction with the semi-conservative replication which the chromosomes showed, was in such remarkable agreement with Watson and Crick's proposals for the structure and mode of replication of the DNA molecule, that it appeared highly probable that the DNA of the chromosome was replicating in the way they suggested. The two nucleotide chains of the DNA molecule (or molecules) in the chromosome would then correspond to the two chromosomal sub-units. The inverted polarity of these chains would preclude rejoining after an exchange except between chains of the same polarity (see Fig. 12.4). It thus appears that the chromosome contains one or a number of DNA molecules which replicate semi-conservatively such that all the old nucleotide chains form one sub-unit and all the new

chains another. This conclusion needs to be accepted with reserve until a satisfactory explanation has been found for some contradictory evidence (see § 17.4). Prescott and Bender (1963), using mammalian cells in tissue culture, have confirmed Taylor's discovery that mitotic chromosomes replicate semi-conservatively, and Taylor (1965) has shown that the replication prior to meiosis is also semi-conservative. Moreover, Herreros and Giannelli (1967) have found 288 single and 128 twin exchanges in the chromosomes of human lymphocytes in culture, and have thus confirmed Taylor's discovery that the frequencies of single and twin exchanges are in the ratio of 2:1, with its implication that the two sub-units of the chromosome are structurally different from one another.

§ 12.3 Evidence for semi-conservative replication of DNA

More decisive evidence about the mechanism of replication of DNA molecules was obtained by Meselson and Stahl (1958) using DNA from *Escherichia coli*. Their technique was to cultivate the bacteria for 14 successive cell generations in a medium where the nitrogen was all the heavy isotope ^{15}N, and then abruptly to change to a normal ^{14}N medium. The growth of the bacterial population was exponential and was recorded by cell counts. Samples of about 4×10^9 bacteria were withdrawn from the culture just before the transfer to ^{14}N and at intervals for several cell generations afterwards. The generation time at the temperature of the culture, 36°C, was approximately 50 minutes. The cell contents of each sample were suspended in a concentrated solution of caesium chloride and centrifuged at 44,770 revolutions per minute for 20 hours. After this time the opposing processes of sedimentation and diffusion had set up a continuous increase of density in the caesium chloride away from the axis of rotation, and the DNA became concentrated at a point in this gradient which is dependent on its own density. The position of the DNA was recorded by ultraviolet absorption photographs. It had previously been established by centrifuging a mixture of DNA from ^{14}N and ^{15}N bacteria that two separate bands then appeared on the ultraviolet photographs due to the difference in molecular weight.

After one generation in the ^{14}N medium, all the DNA showed a band at a position halfway between the ^{14}N and ^{15}N positions, while after 2 generations, bands were present at this position, and at the ^{14}N position, and they were of equal intensity (see Fig. 12.5). The interpretation of these results is shown in the diagram. It is concluded that the DNA molecules consist of two sub-units containing equal amounts of nitrogen and which survive intact for many generations, and that each daughter molecule receives only one parental sub-unit. This is in precise agreement with the expectations from the Watson and Crick model for DNA replication, although the experiment does not establish that the sub-units are single nucleotide chains. However, Meselson and Stahl took the DNA of intermediate density obtained after one generation in ^{14}N medium, and heated it to 100°C for 30 minutes in the caesium chloride solution. Such heat treatment was thought to separate the nucleotide chains of DNA molecules. When the heated DNA was centrifuged,

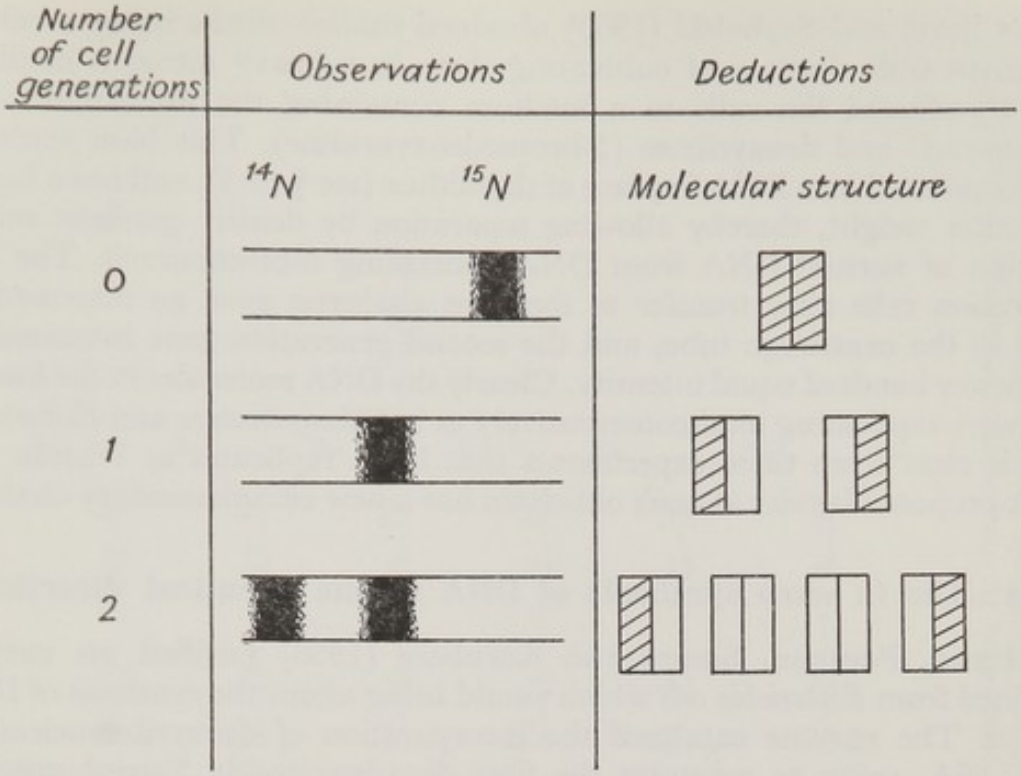


FIGURE 12.5 Meselson and Stahl's observations of the position of the DNA of *Escherichia coli* in a caesium chloride density gradient, after successive cell generations following transfer from a ^{15}N to a ^{14}N culture medium. The deductions concerning the mode of replication of DNA are shown on the right. The rectangles represent the two sub-units inferred to exist within each molecule, open rectangles containing ^{14}N and shaded rectangles ^{15}N.

two bands were obtained instead of the previous one, and moreover they corresponded in position with the bands obtained when pure ^{14}N and pure ^{15}N DNA from *E. coli* were heated and then centrifuged. It was concluded that the DNA of intermediate density consisted of molecules with one heavy and one light polynucleotide chain, and that these were separated by the heat treatment.

Sueoka (1960) applied the density gradient centrifugation technique to a chromosomal organism: *Chlamydomonas reinhardi*. The generation time for mitotically dividing cells at 25°C was about 3 hours. Samples of the culture were removed immediately before transfer from ^{15}N to ^{14}N culture solution, and at 2-hourly intervals for the next 10 hours, and the DNA from each sample was centrifuged in concentrated caesium chloride solution. DNA from *Bos* was used as a density reference. It did not interfere with the observation of the *Chlamydomonas* DNA because of a difference in their densities. The results obtained by Sueoka with *Chlamydomonas* were the same as Meselson and Stahl had obtained with *Escherichia*, namely a single band midway between the ^{14}N and ^{15}N positions after one generation, and two bands of equal intensity, one at this position and one at the ^{14}N position, after two generations. It was concluded that the DNA of *Chlamydomonas* replicates semi-conservatively as in *Escherichia*.

Djordjevic and Szybalski (1960) obtained similar results using a culture of human cells. Instead of cultivating them in a heavy nitrogen medium, they transferred the cells to a medium containing the nucleoside of 5-bromouracil and deoxyribose (5-bromodeoxyuridine). This base analogue is incorporated into DNA in place of thymidine (see § 12.4), and has a higher molecular weight, thereby allowing separation by density gradient centrifugation of normal DNA from DNA containing 5-bromouracil. The first generation cells after transfer to the base analogue gave an intermediate band in the centrifuge tube, and the second generation gave intermediate and heavy bands of equal intensity. Clearly the DNA molecules in the human cells were replicating semi-conservatively as in *Chlamydomonas* and *Escherichia*.

It is clear from these experiments that DNA replicates as Watson and Crick proposed insofar as each old chain has a new complementary chain.

§ 12.4 The *in vitro* synthesis of DNA in one chemical direction

Lehman, Bessman, Simms and Kornberg (1958) purified an enzyme obtained from *Escherichia coli* which would bring about the synthesis of DNA *in vitro*. The enzyme catalysed the incorporation of deoxyribonucleotides into DNA, using as substrate the four deoxynucleoside 5′-triphosphates, that is, of adenine, guanine, cytosine and thymine. Highly polymerised DNA was necessary as a primer and the presence of magnesium ions was also required. The triphosphates needed as substrate had been synthesised by enzymic phosphorylation by adenosine triphosphate (ATP) of the 5′-monophosphates. The existence in *E. coli* of a specific kinase for the phosphorylation of each of the four nucleotides had already been established.

Bessman, Lehman, Simms and Kornberg (1958) showed that in the polymerisation process the omission of any one of the four nucleoside triphosphates reduced the reaction rate to about $\frac{1}{200}$, and omission of the DNA primer abolished it entirely (although it was later shown that some synthesis could occur after a lag period of 3 to 6 hours). They achieved a synthesis *in vitro* of up to 20 times the initial quantity of DNA, and they showed that an amount of inorganic pyrophosphate ions was released equal to the amount of nucleotide incorporated. The synthetic reaction is shown diagrammatically in Fig. 12.6. The necessity for all four nucleoside triphosphates was remarkable and, with the need for DNA as a primer, suggested that synthesis was by a template mechanism such as Watson and Crick had proposed.

Confirmation of the template action of the primer was obtained by Lehman, Zimmerman, Adler, Bessman, Simms and Kornberg (1958) who showed that the base composition of the synthesised DNA was the same as that of the primer. They experimented with DNA from *Aerobacter aerogenes*, *Bos taurus*, *Escherichia coli* and its *T*2 virus, and *Mycobacterium phlei*. The adenine plus thymine contents of the DNA of these organisms, expressed as molar percentages of the total bases, cover a wide range, from 33% for *M. phlei* to 66% for *T*2, and in every instance the product of the polymerase reaction did not differ significantly in base composition from the primer.

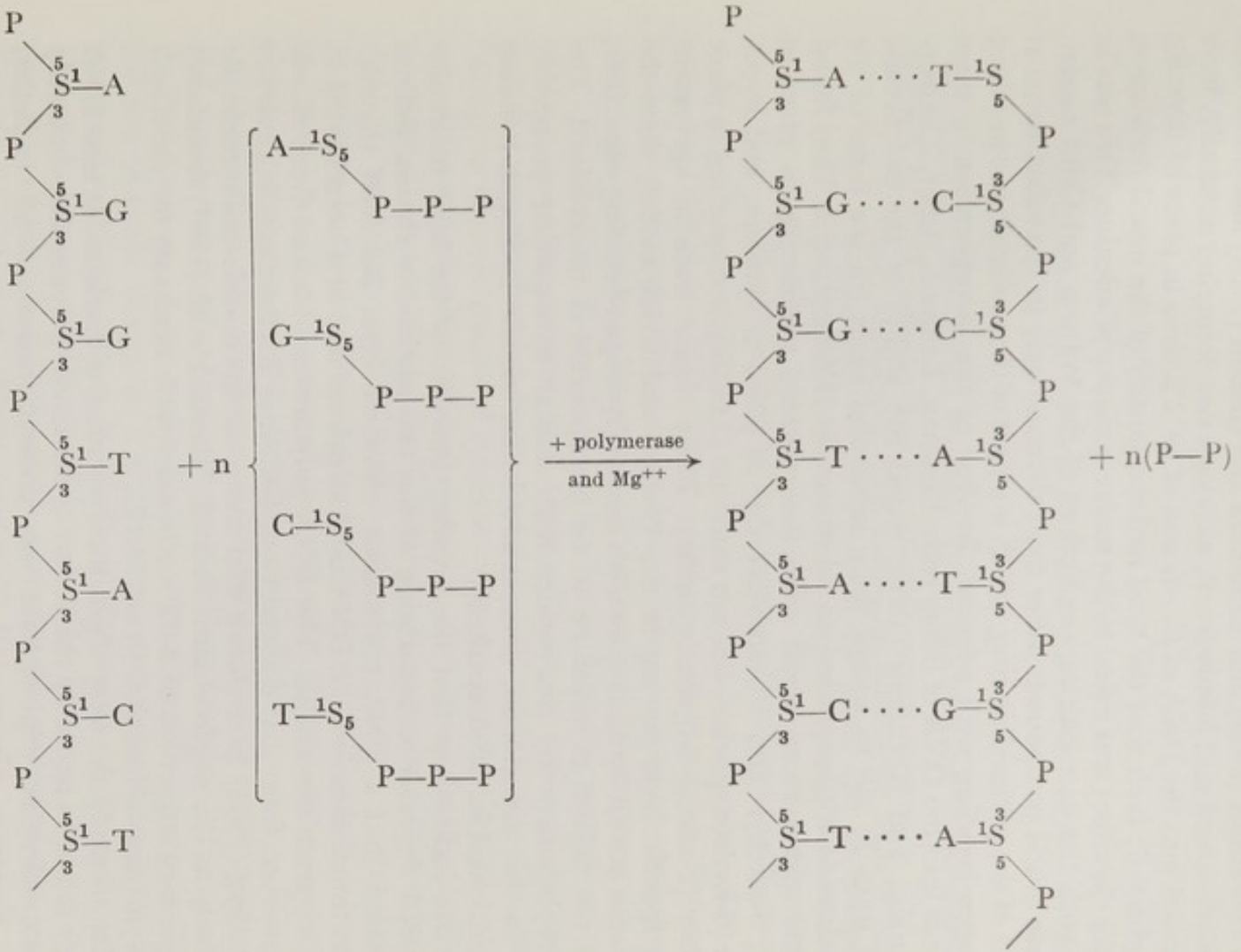


FIGURE 12.6 The synthesis of DNA from the four deoxynucleoside 5′-triphosphates, in the presence of the specific polymerase, magnesium ions, and DNA of high molecular weight as a primer. Inorganic pyrophosphate ions are released in proportion to the number of nucleotides incorporated.
A = adenine, C = cytosine, G = guanine, P = phosphate, S = sugar (deoxyribose), T = thymine.

Moreover the components of each complementary pair of bases on the Watson and Crick model occurred with equal frequency, as in the primer. Variation in the proportions of the four nucleoside triphosphates in the reaction mixture did not affect their proportions in the product, which appeared to be determined solely by their proportions in the primer.

Dunn and Smith (1954) and Zamenhof and Griboff (1954) had discovered that 5-bromouracil and 5-iodouracil, although not occurring naturally, were incorporated into the DNA of *E. coli* and of its *T*2 virus in place of thymine (5-methyluracil) such that the total molar quantity of the uracil derivatives (including thymine) was equal to the molar quantity of adenine. This was in keeping with the base-pairing predictions of the Watson and Crick model, since these uracil compounds differ from thymine only in the substituents at position 5 of the pyrimidine ring, which is a part of the molecule not involved in hydrogen bonding with adenine. In view of the incorporation of these compounds into the DNA of living cells, Bessman, Lehman, Adler, Zimmerman, Simms, and Kornberg (1958) tested the ability of the polymerase obtained from *E. coli* to accept these unnatural bases (in the form of the deoxynucleoside triphosphates) as substrates for DNA synthesis. They found that uracil and 5-bromouracil were incorporated specifically in place of thymine; 5-methyl- and 5-bromo-cytosine in place of cytosine; and hypoxanthine (6-hydroxypurine) but not xanthine (2,6-dihydroxypurine) in place of guanine (2-amino-6-hydroxypurine). These results were in agreement with the specific base pairing in the Watson and Crick model, since the replacements are all by analogues with similar hydrogen-bonding capacities, although the failure of xanthine to be incorporated is unexplained. The specific replacement by nucleotides with similar base-pairing properties, revealed in this experiment, demonstrated that specific hydrogen bonding must be involved in DNA synthesis.

There are indications that the priming function of the high molecular weight DNA depends on separation of the two nucleotide chains. Bollum (1959) found that a DNA polymerase obtained from *Bos* (calf thymus) would not function unless the DNA used as primer had first been heated in order to separate the chains. The DNA polymerase obtained from *E. coli* differs, however, from that derived from *Bos*, since Richardson, Schildkraut and Kornberg (1964) have shown that it can use the double-chain molecule as effectively as the single chains. Indeed, Khorana *et al.* (1967) found that complementary sequences of 8–12 nucleotides were necessary for the *E. coli* DNA polymerase to function (see § 14.12).

In order to study the very slight incorporation of nucleotides into DNA which was found to occur by the action of the *E. coli* polymerase when only one deoxynucleoside triphosphate was present instead of all 4, Adler, Lehman, Bessman, Simms and Kornberg (1958) devised an ingenious technique. The 5′-nucleotide of cytosine, labelled with ^{32}P, was phosphorylated to obtain the nucleoside triphosphate, and then this was incorporated into DNA through the action of the polymerase. Sedimentation coefficients in the ultracentrifuge showed that the incorporated radioactive nucleotides had formed covalent linkages to molecules of the same average size as those

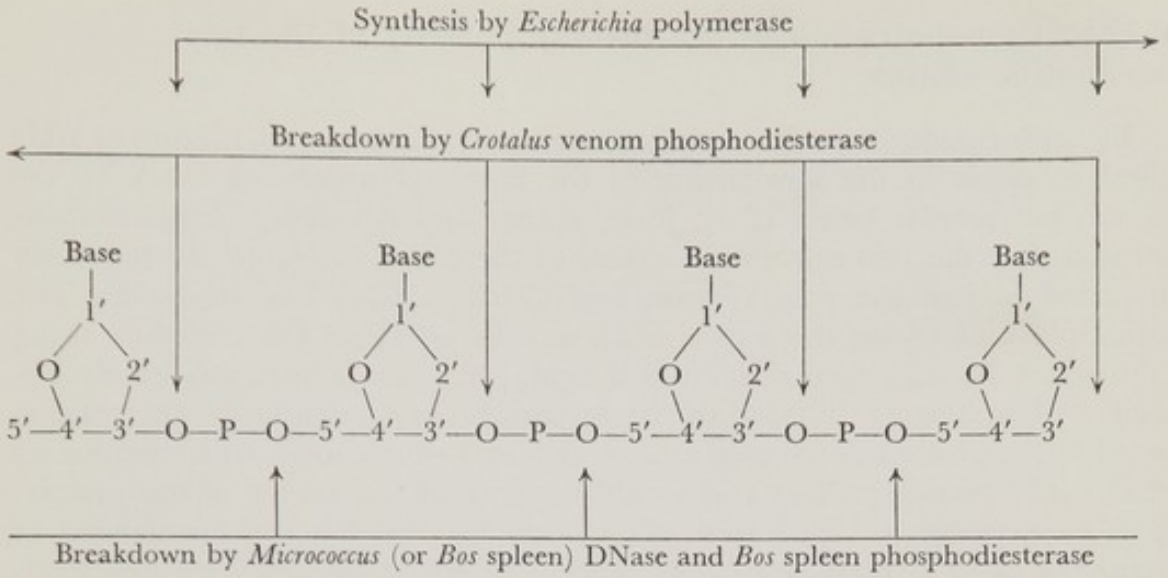


FIGURE 12.7 Diagram to show the action of various enzymes in DNA synthesis and breakdown. The *Escherichia* polymerase adds 5′-nucleotides progressively to the 3′ end of the chain, and the *Crotalus* venom phosphodiesterase reverses this, detaching 5′-nucleotides progressively from the 3′ end. The *Micrococcus* deoxyribonuclease and *Bos* spleen phosphodiesterase cause breakdown into 3′- nucleotides.

of the *Bos* DNA primer. The DNA molecules containing the ^{32}P were hydrolysed, first with deoxyribonuclease from *Micrococcus pyogenes*, and then with a phosphodiesterase from *Bos* spleen. These enzymes break down the molecule into 3′-nucleotides. It was found that all 4 of these 3′-nucleotides (that is, of adenine, guanine, cytosine, and thymine) showed radioactivity, indicating that the phosphate which had entered the DNA molecule attached by a 5′ linkage to the nucleoside of cytosine emerged attached by a 3′ linkage to any of the 4 nucleosides. This showed that the action of the polymerase was to attach 5′ nucleotides to the 3′ position at the nucleoside end (as distinct from the phosphate end) of a nucleotide chain, and to do this irrespective of the base in this terminal nucleotide (see Fig. 12.7). Similar results were obtained when the experiment was repeated using adenine or thymine in place of cytosine in the ^{32}P-labelled 5′-nucleotide.

Confirmation that the polymerase adds nucleotides at the nucleoside end of the chain was obtained by hydrolysis of the product with phosphodiesterase from the venom of the snake *Crotalus adamanteus*. This enzyme hydrolyses DNA nucleotide chains to 5′-nucleotides, and does so stepwise from the nucleoside end of the chain. It was found that nearly all the radioactivity was liberated when less than 3% of the nucleotides had been released, indicating that the venom phosphodiesterase reversed the action of the polymerase (see Fig. 12.7).

Richardson, Schildkraut and Kornberg (1964) have shown that under the action of the *E. coli* DNA polymerase the two chains of DNA act one as template and one as primer, nucleotides being added at the 3′-hydroxyl terminus of the primer chain complementary to those in the other or template chain. It was found that DNA synthesis is inhibited by a 3′-phosphate terminus.

§ 12.5 Demonstration of the inverse polarity of the two nucleotide chains

By an ingenious experiment using essentially the same techniques as were used to establish the mechanism of the *in vitro* synthesis of DNA by the *E. coli* polymerase (see § 12.4), Josse, Kaiser and Kornberg (1961) demonstrated that the two nucleotide chains of the DNA molecule are relatively inverted in just the way Watson and Crick (1953*a*) had supposed. The principle underlying the experiment was to assemble the molecule using 5′-nucleotides and then dismantle it again, but into 3′-nucleotides. Radioactive phosphorus (^{32}P) introduced as part of, say, adenine 5′-nucleotides, would be recovered distributed among all four 3′-nucleotides with frequencies that could be determined and would depend on the source of the primer. The experiments differed from those used to establish the mechanism of synthesis (§ 12.4) in that all four nucleoside triphosphates were provided, with the result that extensive DNA synthesis could occur. In any particular experiment only one of the four bases was associated with the label in the phosphorus of the nucleotide, but each, in turn, was so labelled.

It is conventional to indicate 3′-phosphates with the phosphate symbol (*p*) to the right of the nucleoside symbol and 5′-phosphates with the phosphate symbol to the left. Thus *GpA* stands for *G*3*p*5*A*, where *G* and *A* are the deoxynucleosides of guanine and adenine, respectively. One can imagine the experiment as the passing of the radio-phosphorus from the left hand of one nucleoside to the right hand of the adjacent nucleoside to the left. If the two chains of the molecule 'face the same way', passage of the radio-phosphorus will occur to the neighbour to the left in each chain. In other words, transfer from, for example, adenine to guanine will be expected to occur with the same frequency as transfer from thymine to cytosine ($GpA = CpT$). On the other hand, if the chains 'face opposite ways', passage to the left in each will mean passage in opposite directions physically. Hence, transfer from adenine to guanine would be expected to occur with the same frequency as transfer from cytosine to thymine ($GpA = TpC$). Thus, by determining the relative frequencies with which each base has each of the others to its left as its nearest neighbour, it should be possible to determine whether or not the two chains are relatively inverted. This is on the assumption that the nucleotide sequence is non-random.

Josse, Kaiser and Kornberg obtained such data using, in turn, as primer, DNA from 6 species of bacteria, 5 strains of bacterial viruses and *Bos* (calf thymus). Without exception, the results agreed with the hypothesis that the two chains were relatively inverted, and they contradicted any suggestion that the chains were of similar polarity. A selection of their data is given in Table 12.1, together with some of the data of the same kind obtained by Swartz, Trautner and Kornberg (1962) for a further range of organisms (2 species of plants, 10 species of animals and several more bacterial viruses). The 16 possible combinations of the 4 nucleosides are shown in the first column, and those expected to occur with equal frequency if the two chains are of opposite polarity are paired, and separated by horizontal lines.

Those expected to occur with equal frequency if the chains were of the same polarity are joined by arrows. From the data the percentage frequencies of

TABLE 12.1 Some of the data of Josse, Kaiser and Kornberg (1961) and Swartz, Trautner and Kornberg (1962) for the frequencies of different base sequences in the DNA of various organisms. For explanation, see text.

Base Sequence	*Mycobacterium phlei*	*Aerobacter aerogenes*	*Chlamydomonas reinhardi*	*Homo sapiens*	*Haemophilus influenzae*	*Paracentrotus lividus*
$A + T$	32·6	44.3	46.2	59.5	61·8	64·2
$G + C$	67·4	55·7	53·8	40·5	38·2	35·8
$A3p5A$	2·4 (2·7)	5·9 (4·9)	6·0 (5·3)	9·7 (8·9)	11·6 (9·5)	11·0 (10·3)
$\overline{T5p3T}$	2·6 (2·7)	6·1 (4·9)	5·9 (5·3)	9·7 (8·9)	11·6 (9·5)	10·2 (10·3)
$A3p5T$						
$\overline{T5p3A}$	3·1 (2·7)	5·3 (4·9)	5·4 (5·3)	8·1 (8·9)	9·5 (9·5)	10·4 (10·3)
$T3p5A$						
$\overline{A5p3T}$	1·2 (2·7)	3·6 (4·9)	5·3 (5·3)	6·7 (8·9)	7·3 (9·5)	9·0 (10·3)
$A3p5G$	4·5 (5·5)	5·6 (6·2)	6·0 (6·2)	7·0 (6·0)	5·0 (5·9)	5·3 (5·7)
$\overline{T5p3C}$	4·5 (5·5)	5·7 (6·2)	5·7 (6·2)	7·1 (6·0)	4·9 (5·9)	5·9 (5·7)
$T3p5C$	6·1 (5·5)	5·7 (6·2)	4·6 (6·2)	5·7 (6·0)	5·2 (5·9)	5·9 (5·7)
$\overline{A5p3G}$	6·5 (5·5)	5·8 (6·2)	4·4 (6·2)	6·1 (6·0)	5·4 (5·9)	5·7 (5·7)
$A3p5C$	6·4 (5·5)	5·2 (6·2)	6·0 (6·2)	5·4 (6·0)	4·9 (5·9)	5·8 (5·7)
$\overline{T5p3G}$	6·0 (5·5)	5·2 (6·2)	5·5 (6·2)	4·9 (6·0)	4·8 (5·9)	5·3 (5·7)
$T3p5G$	6·3 (5·5)	6·9 (6·2)	7·3 (6·2)	7·4 (6·0)	6·7 (5·9)	6·7 (5·7)
$\overline{A5p3C}$	6·3 (5·5)	6·7 (6·2)	7·7 (6·2)	7·4 (6·0)	6·7 (5·9)	6·7 (5·7)
$G3p5G$	9·0 (11·4)	6·7 (7·8)	7·1 (7·2)	5·0 (4·1)	3·6 (3·7)	3·3 (3·2)
$\overline{C5p3C}$	9·0 (11·4)	6·5 (7·8)	7·4 (7·2)	4·7 (4·1)	3·7 (3·7)	3·8 (3·2)
$G3p5C$						
$\overline{C5p3G}$	12·2 (11·4)	10·3 (7·8)	9·2 (7·2)	4·3 (4·1)	5·3 (3·7)	3·1 (3·2)
$C3p5G$						
$\overline{G5p3C}$	13·9 (11·4)	8·8 (7·8)	6·3 (7·2)	1·0 (4·1)	3·8 (3·7)	2·0 (3·2)

the two base-pairs have been obtained and are given at the top of the table for each species. From these values the expected frequencies of each of the 16 combinations have been calculated on the assumption that the nucleotide sequence is a random one. These frequencies, as percentages, are given in brackets after each observed percentage frequency.

In addition to the clear indication that the chains are of opposite polarity (equality within the pairs associated in the table, and frequent inequality between the pairs joined by arrows), the data show that each species has a unique pattern of base neighbours. Swartz, Trautner and Kornberg found evidence of fairly consistent differences between bacteria and chromosomal organisms in some of the sequence patterns, notably the low frequency of cytosine to the left of guanine (*CpG*) in chromosomal organisms (illustrated by

Chlamydomonas, *Homo*, and *Paracentrotus* in the table) and the high frequency of guanine to the left of cytosine (*GpC*) in bacteria (illustrated by *Mycobacterium*, *Aerobacter*, and *Haemophilus* in the table). These peculiarities appear to be independent of the guanine-cytosine frequency, which varies widely in different species of both bacteria and chromosomal organisms (see Fig. 14.15). The shortage of *CpG* in chromosomal organisms, particularly mammals, is discussed in § 14.13.

Josse, Kaiser and Kornberg found that, for any particular species, the same sequence patterns were obtained when DNA obtained directly from the organism was used as primer and when the DNA prepared enzymatically was itself used as primer. This indicated that the base sequence was preserved in the replication process. Swartz, Trautner and Kornberg found that a number of different tissues of *Bos* gave the same sequence pattern, and similar results were obtained with *Mus*. It was evident that, like DNA content (§ 11.4), and overall base composition (§ 11.6), the base sequence was species-specific and tissue-independent.

Confirmation that the two nucleotide chains of DNA are of relatively inverted polarity (antiparallel) has been obtained by Chargaff *et al.* (1965) by hydrolysis of the DNA of *Bos taurus*, and isolation of purine dinucleotides and pyrimidine dinucleotides by chromatography. It was found that the sequence *A3p5G* was 1·3 times as frequent as the sequence *G3p5A*, and the inverted complements of these sequences had corresponding frequencies, *T5p3C* being 1·3 times as frequent as *C5p3T*.

§ 12.6 The *in vivo* synthesis of DNA

The mechanism of DNA synthesis shown by the polymerase extracted from *Escherichia coli* by Kornberg and associates, and discussed in § 12.4 and § 12.5, involves the addition of 5′-nucleotides to the nucleoside end of a nucleotide chain. This mechanism alone would not explain how both chains of a DNA molecule could replicate continuously from the same physical end, as Watson and Crick had postulated, since this would require the attachment of nucleotides at the phosphate end of one of the chains. Several authors have found evidence that, at least at the level of resolution provided by the light microscope, or by widely spaced genes, replication *in vivo* can occur in the same physical direction alongside both nucleotide chains.

Cairns (1963) demonstrated this with *E. coli*. His technique was to grow the bacteria in the presence of tritiated thymidine for a known length of time and then find the position of the tritium by gentle extraction of the DNA followed by autoradiography. The film was exposed to the emissions from the tritium for about 2 months before being developed. It was already known that each bacterium in an exponentially growing culture syntheses DNA almost continuously. When bacteria were grown in the presence of tritiated thymidine for 3 minutes, it was found that two pieces of DNA each 60 to 80 μ long were labelled, and about twice this length was labelled with 6 minutes of treatment. At this rate of synthesis, up to about 800 μ of DNA could be

covered in the cell generation time, which at 37° was about 30 minutes. If the DNA was extracted immediately after the labelling the two labelled pieces of DNA were close together, but if the bacteria were allowed to continue to grow for 15 minutes in unlabelled thymidine, the two labelled pieces of DNA were found to be further apart. It was inferred that two labelled chains were being formed simultaneously in the region of replication.

In a second series of experiments, Cairns grew the bacteria in the presence of the tritiated thymidine for a period of 1 hour. This would allow the occurrence of up to two complete replications of the DNA in the presence of the label. The autoradiographs so obtained revealed up to 900 μ of DNA labelled in an unbroken piece. This indicated that the whole of the bacterial DNA occurs in one piece, since assuming it contains two nucleotide chains and has the Watson and Crick structure, this length is in reasonable agreement with previous estimates of the DNA content of *E. coli* nuclei.

Furthermore, whenever a fork was visible, indicating that the DNA had been caught in the act of replicating, one limb of the fork showed twice the density of grains in the emulsion compared with the other limb and with the remainder of the molecule. This is what would be expected if the molecule replicates semi-conservatively, because the first replication in the presence of the tritium would label one nucleotide chain, and at the second replication in the presence of the tritium these labelled and unlabelled chains would separate and a new labelled chain would be laid down alongside each. Hence, at the end of the second replication one daughter molecule would be labelled in one of its chains and the other in both. Different molecules showed the second duplication at various stages of completion and it was evident that the replication was proceeding progressively from one end of the molecule to the other. Some further discoveries made by Cairns by autoradiography of the DNA of *E. coli* are discussed in the next section.

Genetic evidence that, at the level of resolution provided by widely-spaced genes, replication of DNA *in vivo* can occur in the same physical direction alongside both nucleotide chains has been obtained by Yoshikawa and Sueoka (1963) with *Bacillus subtilis*. The frequency of genetic transformation of 10 different characters was studied, using the DNA from an exponentially growing population, and also of a culture in the stationary phase. On the assumption that the cells from the stationary phase have a complete chromoneme*, and not one that has partially replicated, comparison of the relative transforming activity of the DNA from the exponential and stationary growth phases should provide information about the mechanism of replication of the DNA. It was found that a genetic map could be constructed such that the relative transforming activity of a gene near one end was approximately double that of a gene near the other end, with a progressive decline in between. This is what would be expected in an unsynchronized cell population if there was oriented replication from one end of the chromoneme to the other, with both chains undergoing replication and with little interval between the end of one replication and the beginning of the next. Genes near the replication origin would then be represented almost

* Chromoneme = bacterial chromosome—see § 11.1.

twice as often in the DNA of the exponentially growing population as genes near the replication terminus.

Bonhoeffer and Gierer (1963) incorporated 5-bromouracil labelled with radioactive carbon (^{14}C) into the DNA of *Escherichia coli* for varying periods of time, and then studied the distribution of radioactivity in a caesium chloride density gradient. They found that as the average size of the labelled section of the molecule was increased, the radioactivity shifted towards a density of pure hybrid thymine-bromouracil molecules. From quantitative studies, it could be inferred that the chromoneme grew at only one point.

All these results, obtained by various methods, are in agreement with the hypothesis that the DNA in these bacteria replicates progressively from one end to the other by synthesis of a new chain alongside each old one. These experiments have not established, however, whether replication *in vivo* occurs in both chemical directions, that is, by the addition of 5′-nucleotides at the free 3′-hydroxyl end of one chain and at the free 5′-phosphate end of the other. It is possible that DNA synthesis occurs only by the addition of nucleotides to the 3′-hydroxyl termini, as *in vitro*. In at least one daughter-molecule the process would then need to be discontinuous, since the synthesis would be in the direction away from the growing-point. In this way short stretches could be synthesized in the 5′ to 3′ direction and subsequently be connected together by the formation of phosphodiester linkages. Okazaki *et al.* (1968), from study of the length of newly-synthesized chains, favour discontinuous synthesis in both chains, the segments comprising 1000–2000 nucleotides, and Taylor (1968), from study of chromosome replication in *Cricetulus griseus* (Chinese Hamster), believes that large continuous pieces of DNA are assembled by the end-to-end joining of segments 1–2 μ long.

§ 12.7 The mechanism of separation of the two nucleotide chains

From the evidence given in this chapter, there is reason to believe that DNA replicates semi-conservatively by the synthesis of a new chain alongside each old chain. As pointed out in § 12.1, in order to allow the parental nucleotide chains to separate prior to such replication, it would be necessary to break the hydrogen bonds between the complementary bases and to uncoil the chains. The breaking of the comparatively weak bonds would be a simple matter compared with the formidable problem posed by the uncoiling.

Levinthal and Crane (1956) suggested a possible solution. If the molecule rotated about the helix axis the chains could readily separate progressively from one end of the molecule, provided the direction of rotation was anti-clockwise as seen looking down on the end where separation was occurring, and provided the chains as they separated continued to rotate each about its own helix axis. As suggested by Watson and Crick, the molecule was visualized as shaped like a letter Y during the separation process with the crotch of the Y moving progressively downwards. Levinthal and Crane, using data for virus *T*2 of *Escherichia coli* multiplying at 37°C, made estimates

of the viscous drag and of the time taken for separation to occur (100 seconds), and came to the conclusion that the energy required for the rotation of the molecule in order to separate the chains was only about one thousandth of that required for the formation of the new phosphate-sugar bonds in the polymerisation of nucleotides to form the two new chains produced in the replication process. They assumed that the DNA of *T*2 consisted of molecules about 20 μ long (6,000 turns of the helix).

It is now known that the whole of the DNA of virus *T*2 occurs in a single molecule, which measures about 55 μ in length and contains about 150,000 nucleotide pairs, implying 15,000 turns of the double helix. This has been shown by measurement of the length of the thread when gently released from the head of the virus by extraction with phenol or after rupture by osmotic shock. Such measurement can be made either from autoradiographs (Cairns 1961) or from electron micrographs (Kleinschmidt *et al.* 1962; cf. Fig. 11.2). Since the molecule is known to replicate many times in the interval of about 30 minutes between infection of the host and release of the progeny virus, it is evident that an exceedingly efficient uncoiling mechanism must exist. Similar arguments apply to DNA replication in other organisms.

Kuhn (1957) considered that rotatory Brownian motion of the molecular axis was capable of achieving an essential uncoiling of a DNA molecule 3 μ long (900 turns of the helix) within one second and a complete separation of the chains of such a molecule within 50 to 80 seconds. Longuet-Higgins and Zimm (1960) have suggested that in addition there may be a decrease of free energy which would produce a small torque near the axis of the coil at the point of separation in the partly untwisted coil, and so drive the uncoiling.

Cairns (1963) from tritium autoradiographs of intact chromonemes of *Escherichia coli* (see § 12.6) made a quite unexpected discovery: each replicating molecule had the ends of the fork joined (see Fig. 12.8). Furthermore, from the autoradiographs the chromoneme appeared to be circular. Cairns (1964), using a slightly modified technique involving extraction of the tritium-labelled DNA with lysozyme, obtained autoradiographs showing the *E. coli* DNA as an intact circle of circumference approximately 1.1 mm. Usually the DNA was in process of duplication, having the form of two contiguous loops, as in Fig. 12.8.

Yoshikawa, O'Sullivan and Sueoka (1964) discovered that in rapidly growing *Bacillus subtilis* a second DNA replication may begin before the first has been completed. Cooper and Helmstetter (1968) have put forward a general hypothesis to accommodate single or multiple replication points and the observation that there is more DNA per cell in fast-growing cells (see Fig. 12.8).

On the assumption that rotation occurs in separation of the chains, a circular thread with the fork ends joined implies a device at the junction to allow independent rotation of the chains (see Fig. 12.9(*a*)). However, there is as yet no direct evidence from any organism that the chains of the DNA molecule do in fact separate by rotation in the way Levinthal and Crane have postulated.

As already indicated, the 55 μ long chromoneme of virus *T*2 and the

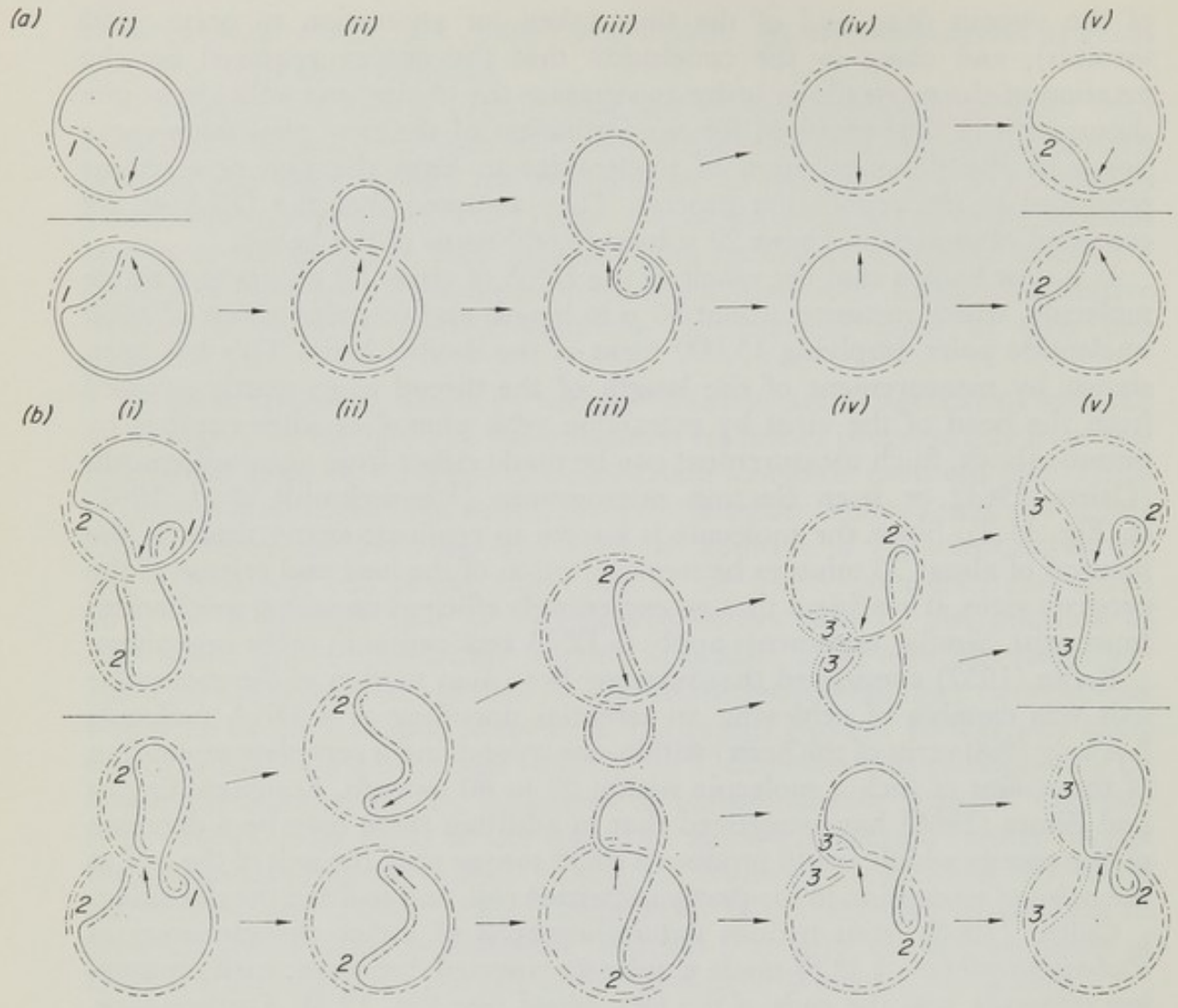


FIGURE 12.8 Diagrams based on those of Cairns (1964) of stages of replication of the DNA of *Escherichia coli* to illustrate the hypothesis of Cooper and Helmstetter (1968), according to which the DNA replication time is constant at 40 min and the interval between the end of replication and the occurrence of cell division is also constant at 20 min, but the cell doubling time and hence the interval between successive DNA replications can vary from 20 to 60 mins.

(*a*) Stages of replication at $12\frac{1}{2}$ min intervals ((i)–(v)) when the doubling time is 50 min.

(*b*) Stages of replication at $6\frac{1}{4}$ min intervals ((i)–(v)) when the doubling time is 25 min.

The numbers 1–3 show the growing-points of successive replications. An unbroken curved line indicates a nucleotide chain synthesized before the first numbered replication, a broken line a chain synthesized at replication no. 1, alternate dots and dashes at no. 2, and a dotted line at no. 3. An unbroken straight line indicates cell division. The cell divisions shown in diagrams (v) occur 20 min after the completion of replication no. 1. The arrows within the circles show the replication origin (and terminus). It seems likely that during replication not more than one nucleotide chain is joined at this point; this would allow rotation of the molecule about the helix axis to occur independently on each side (see p. 202).

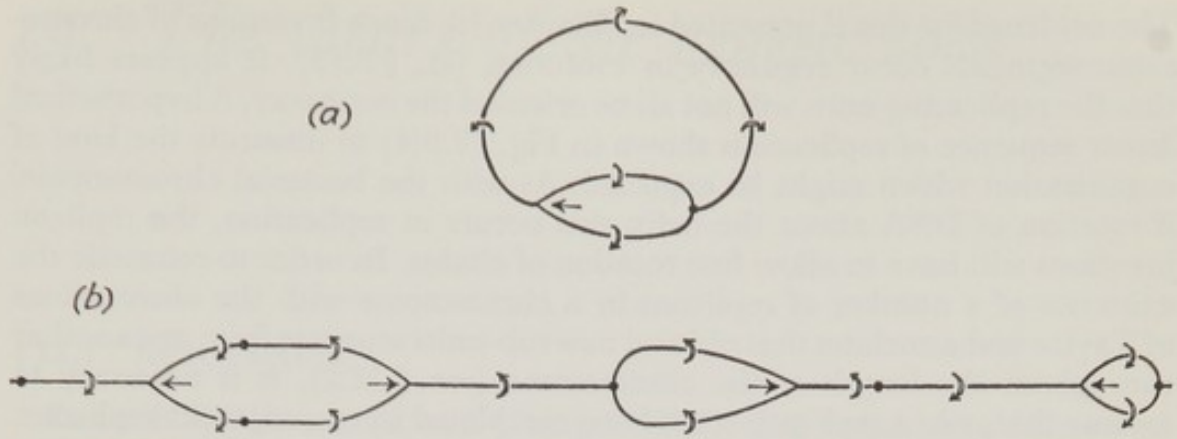


FIGURE 12.9 (*a*) Chromoneme of *Escherichia coli* with its single replicon.
(*b*) Part of a chromosome showing four replicons at various stages of replication.
Dots indicate replicon junctions, where chains can rotate independently of one another. Curved arrows show the direction of rotation and straight arrows the direction in which chain separation and synthesis are occurring.

1100 μ long chromoneme of its host, *E. coli*, each constitute one replicating unit. Jacob and Brenner (1963) proposed the term *replicon* for such a unit of replication. It is established on good evidence that a fragment of a chromoneme, transferred to another cell (see § 16.3), is unable to replicate but may recombine with the intact chromoneme of the acceptor cell. Jacob and Brenner suggested that the capacity of chromonemes to replicate only as a whole and not when fragmented implies the presence and activity of specific determinants controlling replication (see § 15.9).

Chromosomes contain so much more DNA than the chromonemes of bacteria and viruses that it is not surprising that the replication mechanism appears to be differently organised. If the whole of the DNA in a chromosome consisted of one molecule it would have a length of many centimeters. It appears that a chromosome does not replicate progressively from one end to the other. This was first demonstrated by Taylor (1960) with cultured cells of *Cricetulus griseus*. It has been particularly clearly shown by Schmid (1963) with human cells in tissue culture. Each of the chromosomes has its own specific pattern of early and late replicating regions, as revealed by tritium labelling. Evidence for the constancy of these regions at the molecular level was obtained by Mueller and Kajiwara (1966). Using human cells in culture, synchronized in division by amethopterin treatment, they labelled with ^{14}C-ribosylthymine the DNA which replicated early; at a subsequent division they again labelled the early-replicating DNA, but this time with 5-bromodeoxyuridine. The heavy DNA containing the 5-bromodeoxyuridine was separated from the remainder by density-gradient centrifugation and found also to contain the ^{14}C, evidently in the other chain. This establishes that the early- and late-replicating segments are constant in position at the molecular level. This suggests that a chromosome may be made up of a series of replicons. Keyl (1965*a*, *b*) and Pelling (1966) have suggested that the molecular unit of replication in the chromosome is the chromomere.

The evidence for this is presented in Chapter 18. Since inversions of chromosome segments occur regularly in evolution (cf. § 8.15), it appears likely that the replicating units will not all be oriented the same way. A hypothetical linear sequence of replicons is shown in Fig. 12.9(*b*) to illustrate the kind of organization which might be expected. As with the bacterial chromoneme, if rotation of DNA about the helix axis occurs in replication, the replicon junctions will have to allow free rotation of chains. In order to reconcile the existence of a number of replicons in a chromosome with the observations of Taylor and associates that old and new sub-units separate from one another throughout the length of the chromosome (see § 12.2), it is necessary to assume that, when replication has been completed in two adjacent replicons, old chain is linked to old chain and new to new across the replicon junction. The simplest way in which this linking could be maintained would be if the old chains were of one polarity and the new of the other.

Cairns (1966) treated human cells in culture with tritiated thymidine and then extracted the DNA using the same technique as for bacteria. From the autoradiographs he concluded that the replicating units were joined in series end-to-end. Huberman and Riggs (1968) confirmed and extended these observations using cells of *Cricetulus griseus* and *Homo sapiens* in culture. They made the unexpected discovery that the replicons appeared regularly to be in pairs back-to-back, as at the left-hand end of Fig. 12.9; that is, replication appeared to be initiated simultaneously at a common point of origin between the two replicons and then to proceed (at not more than 2·5 μ/min) in opposite directions along each. Before replication begins one chain of the DNA is presumably cut at each replicon terminus as well as at the shared origin, in order to allow the rotation needed to separate the chains (see Fig. 12.9).

In Chapters 17 and 18 further aspects of chromosome structure from the point of view of replication will be discussed.

13. *The theory of the genetic code*

§ 13.1 Introduction

Zamenhof, Brawerman, and Chargaff (1952) had found large differences in the base composition of the DNA of different species of bacteria, and this led them to suggest that there were specific differences in the sequences of nucleotides along the polynucleotide chains of DNA molecules. This idea received strong support from Watson and Crick (1953*b*), who regarded the precise sequence of the bases as the code which carried the genetical information. Their proposed replication mechanism for DNA was admirably adapted to maintain the same order of the bases in the daughter-molecules.

Dounce (1952) and, apparently independently, Gamow (1954) had the brilliant idea that the linear sequence of nucleotides in nucleic acids was responsible for determining the linear sequence of amino-acids in the polypeptide chains of protein molecules. That the linear construction of the genetic material might reflect the linear structure of specific polypeptides was a highly original idea of attractive simplicity. Moreover, it accommodated so well the one gene:one enzyme hypothesis, since all known enzymes are proteins. Gamow drew an analogy between polynucleotides as a long number written in a four-digital system (the four bases) and polypeptides as long words based on a 20-letter alphabet (the 20 different kinds of amino-acids), and he raised the question of how the four-digital numbers could be translated into such words.

Dounce and Gamow's idea has been variously called the sequence hypothesis, the co-linearity hypothesis, and the theory of the genetic code.

§ 13.2 The linear construction of the gene

One of the first questions raised by the co-linearity hypothesis is how the gene is constructed. It had been known ever since Sturtevant (1913) discovered that crossover frequencies fitted a linear map, that the genes appeared to be arranged in a line along the chromosome (§ 7.3). Did this linear construction extend to the intragenic organization?

Several instances of recombination between alleles had been discovered in *Drosophila melanogaster* (see § 9.11). These suggested that the classical bead hypothesis of the gene might not be valid. However, little significant information about the detailed structure of the gene was obtained until the advent of selective techniques for the recovery of recombinants. Such techniques were made possible by Beadle and Tatum's discovery of fungal mutants with specific nutritional requirements (§ 10.2). If two mutants

are very closely linked and have the same biochemical requirement for growth, recombinants could be detected even if of very low frequency by crossing the mutants and germinating the progeny spores on minimal medium, since neither parental type of spore would be able to grow.

Roper (1950) applied this technique to 3 biotin-requiring mutants (bi_1, bi_2, bi_3) of *Aspergillus nidulans* which had been independently obtained by X-ray treatment, and found that crosses of one with another gave recombinants not requiring biotin (prototrophs) with frequencies of about 1 per 2,000 (bi_1 and bi_2) and 1 per 5,000 progeny (bi_1 and bi_3). It was deduced that the biotin-independent progeny were arising by recombination and not by back-mutation because of their genotype for linked character-differences. Furthermore, no prototrophs were obtained from crosses between strains with the same biotin alleles. The three mutants all appeared to be concerned with the same biochemical step, since they each required biotin or desthiobiotin for growth and did not respond to pimelic acid. Moreover, Roper (1953) found that heterokaryons obtained from pairs of them were still biotin-requiring. In other words, each mutant was unable to supply the other's deficiency. Similarly, rare diploid strains of *Aspergillus* which Roper obtained by a selective technique from the heterokaryons, were also biotin-requiring. These had one biotin mutation in one chromosome and the other in the homologous chromosome. By the definition of multiple alleles given in § 9.1, these mutants would qualify as allelic, since they are functionally similar, do not complement one another, and are due to mutations in essentially the same region of the linkage map. However, as with the *Drosophila* examples discussed in § 9.11, the discovery of recombination between such mutants raises the question of what is meant by the terms gene and allele. Pontecorvo (1952*b*) tentatively concluded that the biotin-requiring strains were due to mutations at different sites distinguishable by recombination but within a single gene. On this supposition the gene as a unit in physiological action is a larger chromosome segment than either the unit of mutation or of recombination.

Pritchard (1955) investigated 7 closely-linked and apparently allelic adenine-requiring mutants of *Aspergillus nidulans* which had been obtained independently of one another. Twelve of the 21 possible combinations of the 7 alleles in pairs were tested for recombination by crossing the mutants and plating the progeny ascospores on medium free of adenine. In every instance adenine-independent progeny were obtained. Their frequencies depended on the alleles crossed and ranged from 1 in 1250 to 1 in over 150,000 progeny. Marker genes (*y*, yellow conidia, and *bi*, biotin-requiring), linked to the adenine locus on either side, enabled the behaviour of the neighbouring parts of the chromosome to be followed and confirmed that the prototrophs were arising by recombination and not mutation. Moreover, tests established that the mutants were stable and showed mutation back to adenine independence only with very low frequency. A majority of the adenine prototrophs had a particular crossover genotype for the marker genes, such as would be expected if crossing over had occurred between the alleles. For example, by crossing a strain having adenine-8 and yellow conidia with a

strain having adenine-11 and biotin requirement, heterozygous asci of constitution $\frac{y\ 8\ +}{+\ 11\ bi}$ were obtained, where 8 and 11 represent the adenine alleles and plus (+) stands for the appropriate normal allele (green conidia, biotin independence) of the marker genes. A majority of the adenine-independent ascospores from this cross had the genotype *y bi* for the markers. It was deduced that the *ad*8 and *ad*11 sites of mutation were placed such that the genotype of the diploid nuclei in the young asci could be rewritten $\frac{y\ +\ 8\ +}{+\ 11\ +\ bi}$. Conversely, the most frequent class of adenine independent strains derived from asci of genotype $\frac{y\ 8\ +}{+\ 10\ bi}$ had the + + genotype for the markers (green conidia, biotin independent), from which it was inferred that the formula for the diploid ascus nuclei was $\frac{y\ 8\ +\ +}{+\ +\ 10\ bi}$, with allele no. 10 to the right of allele no. 8. In this way a map giving the sequence of the 7 alleles was constructed. A map was also prepared from the recombination frequencies, both between the alleles and with the markers. The various mapping data were reasonably consistent with one another, and pointed to a linear construction for the adenine gene. Moreover, it was evident that the allele map was collinear with the gene map; for instance, the analysis above indicates a single linear structure with the sequence *y* 11 8 10 *bi*.

§ 13.3 The cis-trans position effect

Roper and Pritchard (1955) had obtained a diploid strain of *Aspergillus nidulans* heterozygous for two adenine alleles (nos. 8 and 16). It had been obtained from a heterokaryon of the two mutants and hence had one allele in one chromosome and the other in the homologous chromosome. Its genotype was $\frac{y\ +\ 8\ +}{+\ 16\ +\ bi}$, using the same notation as previously. This diploid, as expected with allelic mutants, was adenine-requiring. However, by attempting to grow conidia from it on a medium lacking adenine it was possible to select for adenine-independence among them. About 1 conidium in 10^7 was found to show such independence, and from the behaviour of the linked character-differences (*y*, *bi*) it was evident that a process of mitotic crossing-over had taken place. Somatic crossing-over had been discovered by Stern (1936) as a rare occurrence in *Drosophila melanogaster*, and was found to occur between two strands at the four-strand stage (cf. § 8.6), but not to be associated with any reduction in chromosome number. From crosses involving the adenine-independent diploids, Roper and Pritchard found that some of them had the genotype $\frac{y\ +\ +\ bi}{+\ 16\ \ 8\ +}$, such as would be expected if reciprocal recombination had occurred between the alleles. This genotype was identified by the recovery of both *ad*8 and *ad*16 from the progeny. These alleles could be distinguished because *ad*16 allowed slow growth on minimal medium while *ad*8 failed to grow.

From this experiment it was evident that the $\frac{+\ +}{16\ 8}$ genotype (with the two normal alleles in one chromosome and the two mutations in the homologous chromosome) does not require adenine for growth, while the $\frac{+\ 8}{16\ +}$ genotype (with one mutation in each chromosome) does. This was an example of position effect, but it differed from Sturtevant's classical example of Bar eye in *Drosophila melanogaster* in that it did not involve a duplication of a segment of the chromosome (see § 9.10).

Haldane (1941), using a chemical analogy, introduced the terms *cis* and *trans* for the two geometrically isomeric types of double heterozygote previously described by Bateson's terms coupling and repulsion, respectively. Pontecorvo (1950) applied these terms to closely-linked mutations, where differences in the appearance of individuals with the cis and the trans configurations might be expected. Such differences had been discovered by Lewis (1945) with the *star* eye characters in *Drosophila melanogaster* described in § 9.11. He had found that flies with the double mutant in one chromosome and the normal alleles in the other $\left(\frac{S\ \ s}{+\ +}\right)$ had eyes similar to dominant Star $\left(\frac{S}{+}\right)$ and appreciably larger than those of the repulsion heterozygote $\left(\frac{S\ +}{+\ s}\right)$ with its very small and rough eyes. A classic example of the same phenomenon was due to the work of Green and Green (1949) with 3 recessive *lozenge* alleles of *D. melanogaster*. They had followed up Oliver's discovery of recombination between alleles at this locus (see § 9.11). Denoting the 3 alleles by 1, 2, and 3, Green and Green showed that flies of genotype $\frac{+\ +}{1\ 2}$, $\frac{+\ +}{1\ 3}$, or $\frac{+\ +}{2\ 3}$ had normal eyes whereas flies of genotype $\frac{+\ 2}{1\ +}$, $\frac{+\ 3}{1\ +}$, or $\frac{+\ 3}{2\ +}$ had the lozenge characters of glossy eyes with less pigmentation than normal. Several other examples of this cis-trans position effect were discovered in *Drosophila* shortly afterwards, and they included the classic example of multiple alleles, namely, the white eye locus (*w*). MacKendrick and Pontecorvo (1952) and Lewis (1952) found small numbers of recombinants with wild-type (red) eyes from crosses between certain pairs of alleles at this locus. All the red-eyed flies showed recombination for marker genes on either side of *w*. It thus began to appear as if recombination between alleles, together with the cis-trans position effect, might be normal features of the gene.

§ 13.4 The cistron

Genetic recombination in viruses was discovered by Delbrück and Bailey (1947) and by Hershey (1947) with the *T*-even phages of *Escherichia coli*. Hershey and Rotman (1948) studied recombination with a particular

class of mutants which had arisen spontaneously in virus *T*2. In these mutants the clear areas (plaques) produced by the destruction of the bacteria were larger and with a sharper margin than those caused by normal (wild-type) virus. They were called *r* mutants because breakdown (lysis) of the host cells was more *rapid* than normal. Hershey and Rotman found that when a mixture of two *r* mutants of independent origin was added to a liquid culture of the bacterial host (strain *B*), such that there were about 5 times as many virus particles of each kind as there were bacterial cells, many of the bacteria were simultaneously infected with both strains of virus. When fresh bacteria were infected with the progeny virus particles released by the breakdown of the cell-walls of the doubly-infected bacteria, besides many mutant plaques, a certain number of small rough-edged (wild-type) plaques were produced. These had evidently arisen by recombination, because each *r* mutant alone gave none. Moreover, their frequency of occurrence was characteristic for each pair of mutants. From the recombination frequencies, the mutants could be grouped into two classes, subsequently called I and II. There was much closer linkage within each class than between them (see Fig. 18.3). It was remarkable that every mutant tested by 'crossing' (mixed infection) with another gave rise to recombinants. Eleven *r* mutants were studied in this way, and 47 out of the 55 possible crosses were made. Moreover, there was some indication that the recombination frequencies might fit a linear map.

It was subsequently found that the *r*I and *r*II classes of mutants (and also a third group) could be distinguished by their behaviour on other strains of *E. coli*. In particular, the *r*II mutants, unlike *r*I and *r*III, were found not to grow on a strain called *K* of *E. coli*. This inability was taken advantage of by Benzer (1955), since it allowed selection for wild-type recombinants between any two of the mutants, in much the same way as Roper (1950) had selected for recombinants between biotin-requiring mutants of *Aspergillus nidulans*. Benzer used virus *T*4 and crossed 8 *r*II mutants of independent origin in 23 of the 28 possible pairs. Crossing was done by infecting strain *B* of *E. coli* with an equal mixture of the two virus mutants, such that in total there were 6 times as many virus particles as bacterial cells. The progeny virus particles were tested for their ability to multiply on strain *K* of the host. Although neither parent can propagate on *K*, a small proportion of the progeny from every cross produced plaques. The frequency of these was, nevertheless, much higher than could be attributed to back-mutation, and it was concluded that the plaques on *K* were arising through recombination. This was confirmed by the finding that the recombination frequencies from the various crosses gave a reasonable fit with a linear map. The sites of the 8 mutations were found to be in the sequence

47 104 101 103 105 106 51 102

where the figures are the identification numbers of the mutants. These results are what might have been expected from Hershey and Rotman's earlier findings with *T*2 virus using the original non-selective technique.

Benzer tested whether the *T*4 mutants which he had mapped were allelic,

by making mixed infections of them in pairs on strain *K* of the host. Although none of the mutants alone would propagate on *K*, he found that certain combinations of them could supply each other's deficiency and so multiply and produce plaques. When strain *K* is infected with an *r*II mutant, the virus is adsorbed to the host and the bacterial cells are killed, but no progeny virus is produced. With a mixed infection by two *r*II mutants, a situation comparable to the *Aspergillus* heterokaryons studied by Pontecorvo and associates evidently prevails, with two dissimilar hereditary contributions within one cell. When Benzer classified the behaviour of the *r*II mutants by this test for allelism, he made a remarkable discovery. All combinations in pairs of the 6 mutants to the left on the map, that is, nos. 47, 104, 101, 103, 105, and 106 failed to produce plaques, and were evidently alleles (see Fig. 13.1(*f*)). He called this part of the map segment *A*. Similarly, the other two mutants, nos. 51 and 102, also failed to complement one another (Fig. 13.1(*g*)): they formed segment *B*. But any mutant in segment *A* when paired with either of the *B* mutants produced plaques on strain *K* of the host (Fig. 13.1(*h*)). There thus appeared to be two segments within the *r*II region corresponding to independent functional units.

If *K* bacteria were infected with a mixture of wild-type virus and an *r*II mutant (Fig. 13.1(*a*) and (*b*)), the virus was found to multiply and both types appeared in the progeny. It was evident that the *r*II mutants were recessive to the normal condition, and that the wild-type virus could supply whatever was needed to allow the *r*II mutants to multiply. It was presumed that the *cis* configuration of a double mutant and wild type (Fig. 13.1(*c*)–(*e*)) would similarly allow multiplication to occur, irrespective of the position on the map of the mutant.

The situation which Benzer had found in virus *T*4 was evidently similar to that known for several genes in *Drosophila* and *Aspergillus*, with recombination between alleles and a cis-trans position effect. The *r*II mutants were particularly interesting, firstly, because of the proximity of two functional units, mutation of either giving the same phenotype, and secondly, because of the neat way in which the recombination and complementation data established this. Benzer suggested that the *A* and *B* segments might affect sequential steps in a chain of synthesis. Alternatively, the two segments might each control the production of a specific polypeptide chain, the two chains later being combined to form an enzyme. With either of these explanations, the end-product of the action of the two segments would be a single substance, the lack of which was presumed to cause the *r*II phenotype.

Benzer (1955, 1957) found that certain *r*II mutants gave no detectable wild-type recombinants with any of several other mutants, which nevertheless gave wild-type recombinants with each other. Such mutants can be represented as occupying a segment of the linkage map rather than a point. It was significant that back-mutation of such a mutant had never been observed, whereas some of the point mutations showed reversion to wild-type not infrequently. The most likely explanation for both the recombination behaviour and the stability of these mutants appeared to be that they had originated through the loss of a segment of the hereditary material covering

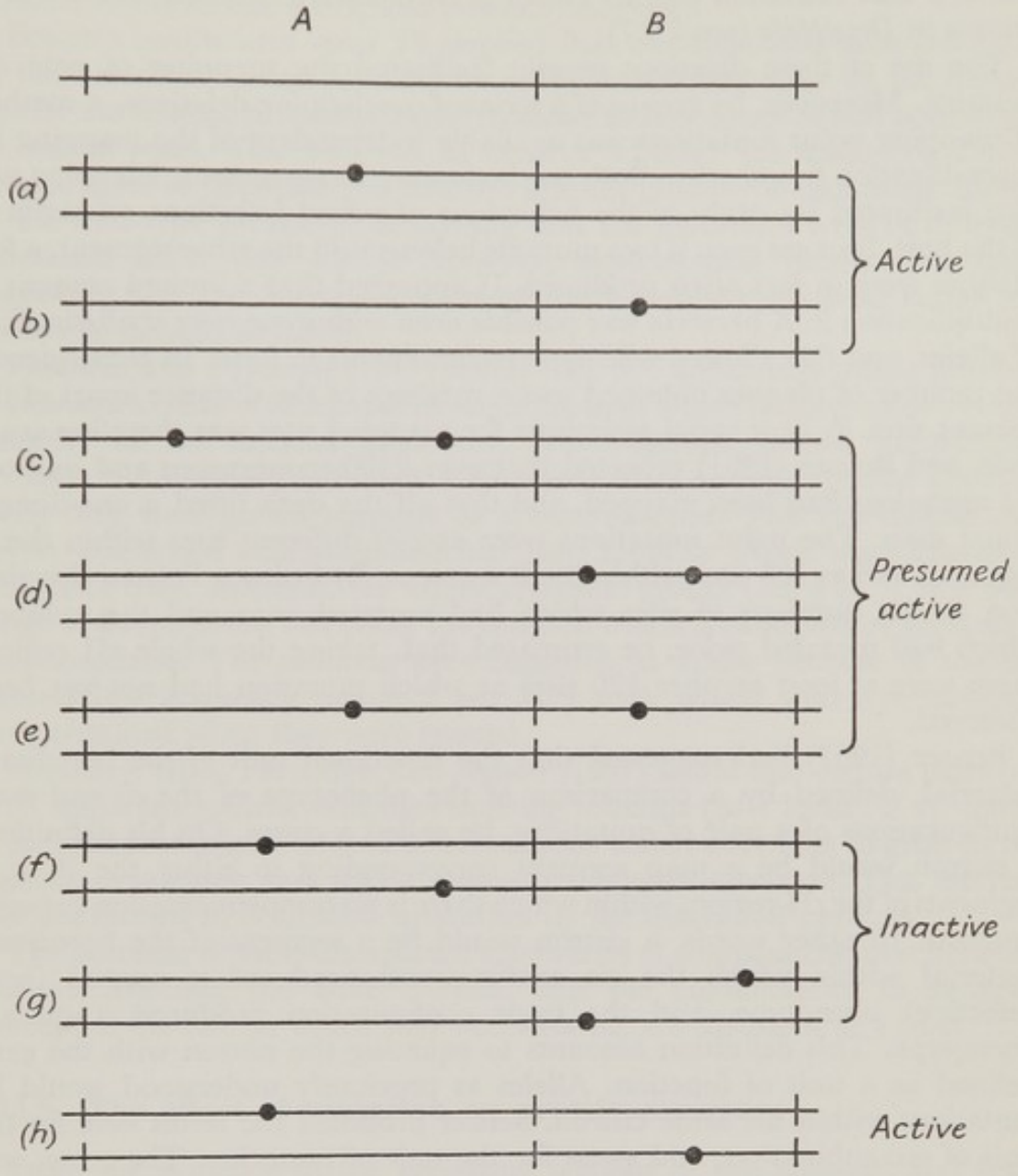


FIGURE 13.1 Diagram to show Benzer's results from mixed infection of *Escherichia coli* strain *K* by two strains of virus *T*4. The dots show the position of the *r*II mutations on the linkage map. 'Active' means that extensive virus multiplication took place.
(*a*), (*b*) Single virus mutants mixed with wild-type virus.
(*c*)–(*e*) Double mutants mixed with wild-type (that is, cis configuration).
(*f*)–(*h*) Mixed infection by two single mutants (that is, trans configuration).
A and *B* are the two cistrons inferred to be present.

the length which they appeared to occupy on the linkage map. It will be recalled that mutation due to loss of a chromosome segment was already known in *Drosophila* (see § 9.7).

The use of these deletions greatly facilitated the mapping of new *r*II mutants. Moreover, by means of a series of overlapping deletions, a method of mapping point mutations was available independent of the mapping by recombination frequencies. Both methods were in agreement. Mapping was also facilitated by study of the behaviour of mixed infections on strain *K* of the host, because even if two mutants belonged to the same segment, a few plaques were in fact often produced. It appeared that a limited amount of multiplication in *K* bacteria was possible even with some *trans* configurations of alleles, and this allowed wild-type recombinants to form. In consequence, the number of plaques obtained was a measure of the distance apart of the mutant sites. A very rapid technique for mapping sites was therefore available, and Benzer (1961) reported that over 2400 spontaneous and induced *r*II mutations had been mapped, and that all the data fitted a one-dimensional map. The point mutations were at 200 different sites within the *A* segment, and at 108 sites within the *B* segment. By fitting a Poisson distribution to the numbers of sites which had mutated once and the numbers which had mutated twice, he estimated that, taking the whole *r*II region, there were at least another 120 sites at which mutation had not yet been observed.

Benzer (1957) had suggested that the functional unit of the hereditary material, defined by a comparison of the phenotype of the *cis* and *trans* configurations of a pair of mutations, be called a *cistron*. On his definition, a cistron would be a map segment corresponding to either the *A* or *B* segments of the *r*II region, within which there is no complementation between mutants. In other words, a cistron would be a segment of the hereditary material within which the cis configuration produced a normal (non-defective) phenotype, and the trans configuration produced a mutant phenotype. This definition amounts to equating the cistron with the gene defined as a unit of function. Alleles as previously understood would be mutations within the same cistron. Benzer proposed the terms *recon* for the unit of recombination, and *muton* for the unit of mutation. The recon was defined as the smallest element in the one-dimensional array that is interchangeable (but not divisible) by genetic recombination, and the muton as the smallest element that, when altered, can give rise to a mutant form of the organism. The introduction of these terms was a recognition that the bead hypothesis was inadequate, and that the term gene, hitherto used more or less indiscriminately for the unit of function, of recombination, and of mutation, was insufficiently precise.

Benzer drew attention to the remarkable parallelism between genetic maps, which appear to be one-dimensional at all levels of analysis, and the one-dimensional character of DNA. On the basis of (*a*) the total length of the linkage map for all the mutant characters in virus *T*4 that had been recognised and mapped, and (*b*) the total content of DNA, which was assumed to have the Watson and Crick structure, Benzer estimated that the *r*II cistrons probably contained several hundred or perhaps a few thousand

nucleotide pairs, whereas the recon and the muton comprised a very small number of nucleotide pairs, and possibly only one.

Benzer's results with virus *T*4 implied that the functional units (cistrons) in the hereditary material could be defined unambiguously by the normal test for the allelism of closely-linked recessive mutants such as had been in use for many years. This test was to observe whether the trans (repulsion) heterozygote or heterokaryon had the mutant phenotype indicating alleles, or the wild-type phenotype indicating that the mutations were in different functional units (see § 9.1). However, experimental results with fungi suggested that the functional units of the hereditary material could not always be defined so easily.

Fincham and Pateman (1957) found that two apparently allelic mutants of *Neurospora crassa* of independent origin, both of which formed no detectable quantity of the enzyme glutamate dehydrogenase, would nevertheless produce the enzyme, although with lower than normal specific activity, when they were grown together as a heterokaryon. This remarkable discovery concerned amination-deficient (*am*) mutants which were unable to synthesize from ammonia the alpha(α)-amino groups (see § 13.5) of a wide range of amino-acids, this being the reaction which is catalysed by glutamate dehydrogenase. The mutants were regarded as allelic because of the extremely low frequency (about 1 per 10^5) with which wild-type progeny were obtained when they were crossed.

Similarly, Giles, Partridge, and Nelson (1957) found that certain pairs out of 21 apparently allelic adenine-requiring mutants (*ad*-4 locus) of *N. crassa*, of independent origin, formed heterokaryons which were able to grow in the absence of adenine and which synthesized adenylosuccinase. This enzyme was not formed in detectable quantities by the individual mutants.

The outcome of these unexpected discoveries of complementation between mutants affecting the same enzyme, and the modifications to Benzer's concept of the cistron which they necessitated, are discussed in § 13.6.

§ 13.5 The effect of mutation on protein structure

From the evidence in the preceding sections of this chapter, and from similar results obtained subsequently for other genes, it appears that the first question raised by the genetic code idea, namely, how the gene is constructed, has been answered. As a unit of function it appears to be a linear structure, and to contain numerous smaller units capable of mutation and of recombination with one another. This is the construction that might be expected if the genetic material is DNA, with nucleotide sequence of paramount importance, and such that alteration of one or more nucleotides at any point incapacitates the whole gene. A second question raised by the Dounce-Gamow hypothesis of amino-acid sequence in polypeptides determined by nucleotide sequence is whether gene mutation can be shown to alter amino-acid sequence.

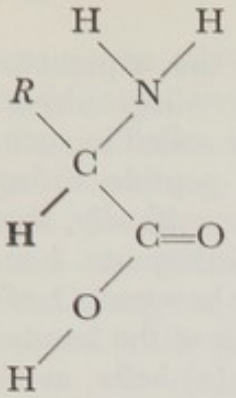
Neel (1949) established that the human disease called sickle cell anaemia is inherited as a Mendelian recessive. This disease is widespread in West Africa and in American negroes (see § 2.6), and is characterized by a severe anaemia

which is usually fatal in childhood. In individuals with the disease, the erythrocytes change their form when the oxygen concentration is low, taking on the shape of a sickle instead of the normal biconcave disc. Neel found that the parents of such individuals were quite healthy, but always had a rather similar condition of the blood called sicklaemia or sickle cell trait. In sicklaemia the crescentic shape of the erythrocytes occurs only at lower oxygen concentrations such as are not encountered in the circulating blood, and there are no anaemic symptoms. From the regular occurrence of sicklaemia in the parents of individuals with sickle cell anaemia, it was inferred that sicklaemia was the heterozygous and sickle cell anaemia the homozygous condition for the sickling gene (cf. Fig. 13.4(*a*)).

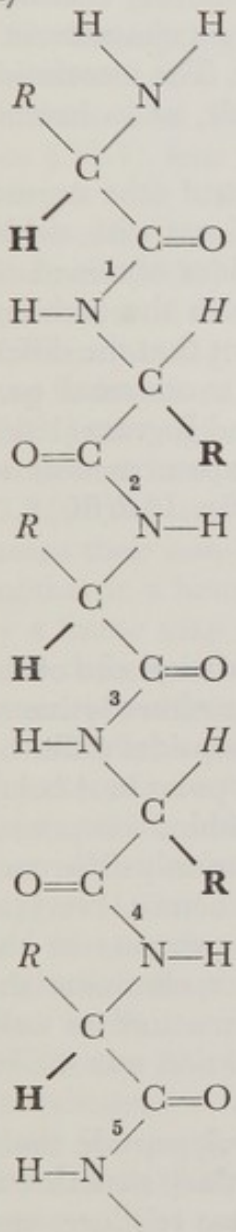
Pauling, Itano, Singer, and Wells (1949) found that the difference between sickling and normal erythrocytes was in their haemoglobins, which showed different electrophoretic mobilities. Moreover, sicklaemia haemoglobin was found to consist of about equal proportions of normal haemoglobin and sickle cell anaemia haemoglobin, as expected of the heterozygote. Ingram (1956) showed that there was a specific chemical difference between the two globins (the protein part of haemoglobin). He had devised a rapid technique for characterising the chemical properties of a protein in considerable detail. Trypsin was used to split the peptide chains of haemoglobin and then the small peptides formed, which numbered about 30, were separated by a two-dimensional combination of paper electrophoresis and paper chromatography. Most of the peptides differ in net charge, and hence migrate at different speeds in an electric field on wet filter paper. The paper is then turned through 90° and a further separation brought about by their differing rates of migration in particular solvents. Finally, the position of the spots corresponding to the different peptides is revealed by staining with ninhydrin. Ingram called the resulting chromatogram the fingerprint of the protein.

Proteins are made up of one or more polypeptide chains. In haemoglobin there are 4, comprising 2 of one kind (alpha(α)-chains) and 2 of another (beta(β)-chains). Polypeptide chains consist of amino-acids linked together in a linear sequence by peptide bonds. In each of the 4 chains of the haemoglobin molecule there are about 150 amino-acids. All amino-acids have a carboxyl group (—COOH), an amino group ($—NH_2$), a hydrogen atom (—H), and a particular radical (—R), attached to one carbon atom called the α-carbon (Fig. 13.2(i)). With 4 different groupings joined to the α-carbon, there are 2 stereo-isomers of each acid (except glycine, where R = —H). Only the L-isomers (Fig. 13.2(i)) ordinarily occur in proteins. The radicals (R) are very diverse in structure and are listed in Fig. 13.2(iii) for the 20 amino-acids normally found in proteins. One of them, proline, is strictly-speaking an imino-acid: the R group is joined at its far end to the α-carbon amino group. In the formation of the peptide bond, amino-acids link together by the attachment of the α-carbon carboxyl group of one to the α-carbon amino group of the next, with the elimination of a water molecule (see Fig. 13.2(ii)). A polypeptide chain will, therefore, have a free amino group at one end and a free carboxyl group at the other.

(i)



(ii)



(iii) *R*

a	—H	*Gly*cine
b	—CH_3	*Ala*nine
c	—CH_2OH	*Ser*ine
d	—$CHOH{\cdot}CH_3$	*Thr*eonine
e	—$CH_2{\cdot}SH$	*Cys*teine
f	—$CH_2{\cdot}CH_2{\cdot}SCH_3$	*Met*hionine
g	—$CH(CH_3)CH_3$	*Val*ine
h	—$CH_2{\cdot}CH(CH_3)CH_3$	*Leu*cine
i	—$CH(CH_3)CH_2{\cdot}CH_3$	*Isol*eucine
j	—$CH_2{\cdot}COOH$	*Asp*artic acid
k	—$CH_2{\cdot}CO{\cdot}NH_2$	*Asparagin*e
l	—$CH_2{\cdot}CH_2{\cdot}COOH$	*Glu*tamic acid
m	—$CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH_2$	*Glutamin*e
n	—$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NH{\cdot}C(NH)NH_2$	*Arg*inine
o	—$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NH_2$	*Lys*ine

p —$CH_2{\cdot}$C$_6$H$_5$ *Phe*nylalanine

q —$CH_2{\cdot}$C$_6$H$_4$OH *Tyr*osine

r —$CH_2{\cdot}$C$_3$H$_3$N$_2$ *His*tidine

s —$CH_2{\cdot}$C$_8$H$_6$N *Try*ptophan

t C$_4$H$_7$NO$_2$ *Pro*line

FIGURE 13.2 Structure of amino-acids and polypeptide chains.
(i) An amino-acid.
(ii) Part of a polypeptide chain. The peptide linkages are numbered from the amino group end of the chain.
(iii) The formulae of the 20 amino-acids normally found in proteins.
In (i) and (ii), bold type and italics indicate projection in front of and behind the plane of the page, respectively.

The primary structure of a protein is determined by the sequence of amino-acids in its polypeptide chain or chains. X-ray crystallography has shown that in proteins the polypeptide chain (or chains) is coiled in such a way that hydrogen bonding can occur between each peptide linkage (—CO·NH—) and the next-but-two in the chain. More specifically, there is hydrogen bonding between the oxygen atom of peptide linkage no. 1 and the hydrogen atom of peptide linkage no. 4, and similarly between O of 2 and H of 5, O of 3 and H of 6, and so on. The numbering of the bonds is from the free amino group end of the chain. This alpha(α)-helix, as the coiled polypeptide is called, forms the secondary structure of a protein. Many proteins, including all those that are active in promoting chemical reactions, have the α-helix folded in an irregular way to give a characteristic shape to the molecule, which is called its tertiary structure. The association of a number of polypeptide chains in one protein molecule, as in haemoglobin, gives rise to a quaternary structure.

Ingram (1956) found that the fingerprint of haemoglobin *A* (the normal molecule) differed from that of haemoglobin *S* (from patients with sickle-cell anaemia) in respect of just one of the 30 or so peptides obtained by trypsin digestion. Trypsin breaks peptide links derived from the carboxyl groups of the amino-acids lysine and arginine. It was evident that the difference between the two haemoglobin molecules was confined to one small part of one of the polypeptide chains. Ingram(1957) and Hunt and Ingram (1959) analysed this peptide and showed that the amino-acid sequences were as follows (the abbreviations refer to the italicized letters in Fig. 13.2(iii)):

A Val–His–Leu–Thr–Pro–Glu–Glu–Lys

S Val–His–Leu–Thr–Pro–Val–Glu–Lys

The histidine is at the amino end and the lysine at the carboxyl end of the peptide. (All peptides formed by trypsin digestion will have either lysine or arginine at their carboxyl end.) It is evident that the two peptides differ by only one amino-acid, the glutamic acid residue at a specific point in *A* being replaced in *S* by valine. In each half-molecule of haemoglobin, comprising nearly 300 amino-acids, this single substitution constituted the only difference between haemoglobins *A* and *S* as regards their primary structure. Previous investigations had failed to establish whether the difference was in the primary or the tertiary structure. Ingram's work did not eliminate the possibility that there might be differences in the tertiary structure as well. However, for the first time, the effect of a single gene mutation was shown to be a change in one amino-acid in a polypeptide chain.

It is tentatively assumed that the tertiary structure of a polypeptide chain is determined exclusively by its primary structure. The tertiary structure is maintained by disulphide (—S—S—) links between cysteine residues, and by chemical or physical bonds between other amino-acid residues. It thus seems possible that the folding to give the tertiary structure could occur largely spontaneously during the synthesis of the polypeptide chain, which is known to occur progressively from the free amino end (see § 14.10). Although the change in the primary structure of haemoglobin due to the sickling

mutation is confined to $\frac{1}{300}$th part of the molecule, its effects are profound. It seems likely that the replacement of 2 charged glutamic acid residues in normal haemoglobin (one in each half-molecule) by 2 uncharged valines alters the charge distribution on the surface of the molecule sufficiently to cause the abnormally low solubility of reduced haemoglobin *S*, which causes the sickling of the erythrocytes in the anaemia. This seemingly trivial change of one amino-acid in each half-molecule would then be wholly responsible for this lethal disease.

§ 13.6 The hybrid-protein hypothesis of allelic complementation

In attempting to explain the complementation between mutants of *Neurospora crassa* affecting the same enzyme, which had been discovered by Fincham and Pateman (1957) and Giles, Partridge and Nelson (1957) (see § 13.4), four observations were of importance. Firstly, and most remarkably, it was discovered that the patterns of complementation between alleles could be represented by a linear map. This was shown by Pateman and Fincham (1958) for *am* mutants, Catcheside and Overton (1959) for *arg*-1 mutants, and Woodward, Partridge, and Giles (1958) for *ad*-4 mutants of *N. crassa* (see Fig. 13.3). The sequence of the mutants on the complementation map is based on the supposition that the mutants have overlapping non-functional regions. These non-functional regions are indicated by the horizontal lines to the right of the tables of complementation data in Fig. 13.3. If two mutants are shown on the map by lines which overlap, this means their non-functional regions overlap and they fail to complement one another in a heterokaryon. The ability to represent allelic complementation by a linear map suggests that there is interaction between linear structures with defects in different regions. These linear structures need not be the genes themselves, but could be products of gene action.

Secondly, complementation between alleles seemed on the whole to be the exception rather than the rule. A majority of allelic mutants, including many which behaved genetically as if they were point mutations rather than deficiencies (cf. § 13.4), were found to be non-complementary. This suggested that the gene could not be subdivided into regions of distinct and non-overlapping function. Instead, it appeared that the linear complementation maps reflected the behaviour of gene products rather than the gene itself.

Thirdly, Fincham and Pateman (1957) found that the level of glutamate dehydrogenase activity through interallele complementation was comparatively low compared with that of wild-type fungus. Woodward, Partridge, and Giles (1958) made a similar discovery with adenylosuccinase-deficient mutants. The enzyme activity rarely exceeded 25% of that of the wild-type.

Fourthly, Fincham (1959*a*) discovered that the enzyme resulting from interallele complementation was qualitatively abnormal. He found that a heterokaryon of alleles nos. 1 and 2 at the amination (*am*) locus produced a peculiar kind of glutamate dehydrogenase. It differed from wild-type enzyme in its low thermostability. Similar results were obtained with

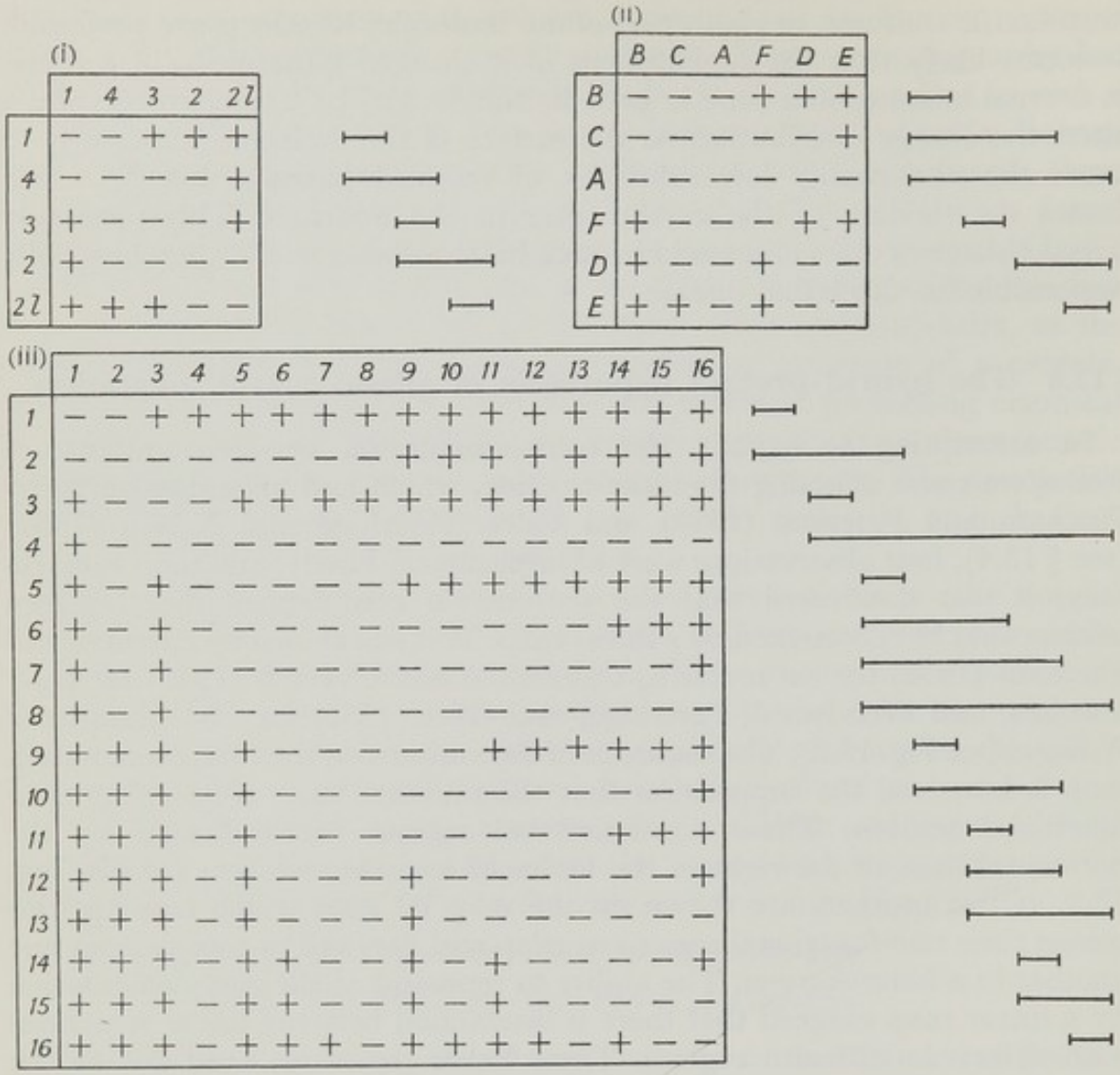


FIGURE 13.3 Results obtained from tests for complementation between allelic mutants of *Neurospora crassa*.

(i) Data of Pateman and Fincham (1958) for 5 alleles at the amination (*am*) locus.

(ii) Data of Catcheside and Overton (1959) for 6 alleles at the arginine-1 (*arg*-1) locus.

(iii) Data of Woodward, Partridge and Giles (1958) for 16 alleles at the adenine-4 (*ad*-4) locus.

The letters or numbers specify the mutants, a plus sign (+) indicates complementation, and a minus sign (−) indicates no complementation. The set of horizontal lines to the right of each table shows the one-dimensional complementation map derived from the data. Non-overlapping lines indicate complementation, and overlapping lines no complementation. In (ii), the sequence of *B* and *F* on the complementation map is arbitrary.

the enzyme produced by the heterokaryon of alleles nos. 1 and 3, and this enzyme had in addition a lower than normal affinity for glutamate.

Fincham and Pateman (1957) suggested that allelic complementation might be due to the partial reconstitution of a component of the enzyme-forming system. It was assumed that two different defective nuclear products interacted in the cytoplasm of the heterokaryon. Giles, Partridge and

Nelson (1957) also favoured nuclear product interaction as an explanation. Catcheside and Overton (1959) suggested that the complementation might be due to the enzyme consisting of an aggregate of two or more normally identical polypeptide chains. This would give an opportunity in a heterokaryon for the chains produced by each mutant to become associated, and through interaction of some kind, to restore the enzyme activity which the individual mutants lacked. They suggested that genes in which all the alleles failed to complement one another might control proteins consisting of only a single polypeptide chain. This possibility is exemplified by the histidine-6 (*his*-6) locus in *N. crassa*, where Catcheside (1960) found no complementation among 95 mutants tested in all pairwise combinations.

Strong support for this hybrid-protein hypothesis of allelic complementation was obtained when Fincham (1959*a*) found that the heterokaryon enzyme was qualitatively abnormal, and the hypothesis was confirmed by the demonstration of complementation *in vitro* between proteins isolated from mutants. Woodward (1959) obtained complementation by mixing extracts from pairs of mutants individually deficient for adenylosuccinase. Fincham and Coddington (1963) found that purified proteins from mutants nos. 1 and 3 at the *am* locus could interact to form a mixture of enzyme types very similar to that found in the heterokaryon of these mutants, and by labelling each mutant protein in turn with radioactive sulphur (^{35}S), Coddington and Fincham (1965) showed that the active interaction product contains sub-units from both mutant proteins, and that the ratio of the two is about 1:1 in at least a part of the hybrid material. The sedimentation characteristics of the hybrid enzyme, studied by sucrose density-gradient centrifugation, confirmed that the hybrid molecules contained the normal number of sub-units.

Clear evidence for the hybrid-protein model of complementation was also obtained by Schlesinger and Levinthal (1963) with mutants of *Escherichia coli* deficient for the enzyme alkaline phosphatase. This enzyme has been shown to consist of two normally identical sub-units, and so can be described as a dimer. Proteins serologically related to alkaline phosphatase were extracted from several mutants and purified. The sub-units of the protein were separated by reduction with thioglycollate in urea. The monomers derived in this way from different mutants known to complement one another *in vivo* were then mixed pairwise, after acidification, and it was found that partially active enzyme was obtained. It was shown by starch electrophoresis, by sedimentation in a sucrose gradient, and from the quantity of enzyme formed with different proportions of the parental monomers, that this enzyme was a hybrid dimer consisting of a monomer from each of the mutant proteins used in the reaction.

It thus appears that allelic complementation results from association between polypeptide chains derived from the two parent strains to produce active enzyme molecules. As Fincham (1959*a*) pointed out, this explanation of complementation means that Benzer's term cistron can be retained as the functional unit of the hereditary material, provided the definition of a cistron is modified such that the trans phenotype has to be non-defective

at the enzyme level before two mutants are recognised as belonging to different cistrons. In other words, two mutants in the trans configuration in a heterokaryon or diploid will give normal enzymes if they are in different cistrons, and abnormal enzyme (or none) if in the same cistron.

§ 13.7 The one cistron:one polypeptide hypothesis

It is known that some protein molecules, such as insulin and the protein part of haemoglobin, contain dissimilar polypeptide chains. Are the amino-

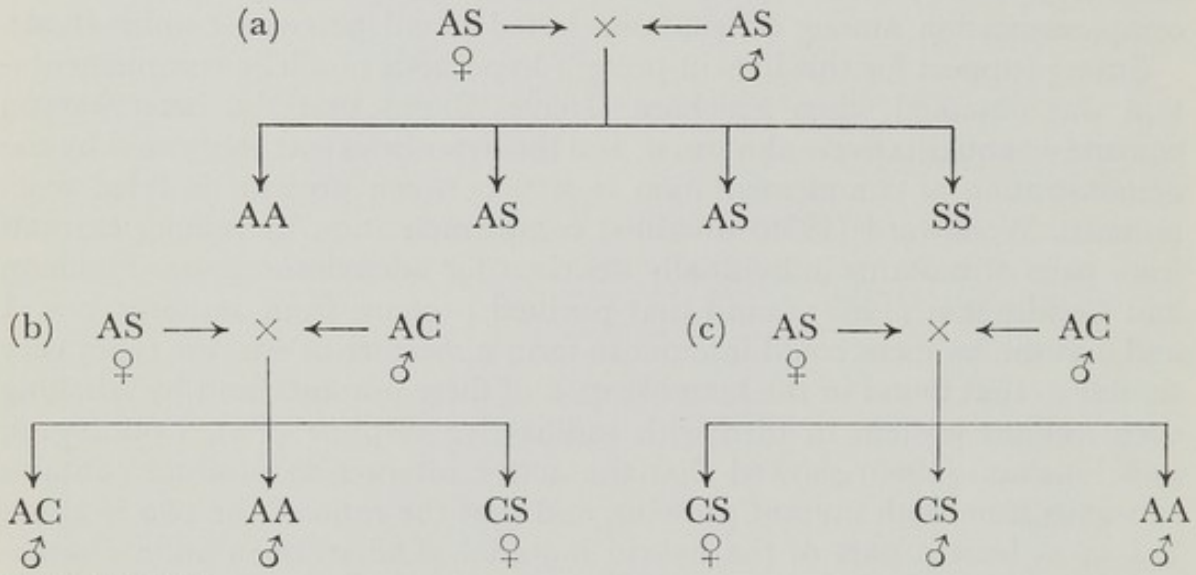


FIGURE 13.4

(*a*) The expectations on Neel's hypothesis of the inheritance of sickle cell anaemia.
(*b*) and (*c*). The two families in which Itano and Neel (1950) discovered haemoglobin *C*.
A, *C*, *S*. Three kinds of haemoglobin. *A*—Normal adult haemoglobin. *S*—Sickle cell haemoglobin. *C*—Another abnormal haemoglobin. The phenotypes of individuals with various haemoglobin combinations are as follows:
AA—Normal.
AC—Normal, but erythrocytes contain haemoglobin *C* as well as *A*.
AS—Normal, but erythrocytes sickle at low oxygen concentrations (sickle cell trait).
CS—Mild form of sickle cell anaemia.
SS—Sickle cell anaemia.

acid sequences in these chains determined by the same cistron or by different cistrons?

Itano and Neel (1950), in studying the inheritance of sickle cell anaemia in man, encountered two families in which there were children with a mild form of the disease. It was found that in each instance only one of the parents had sicklaemia (sickle cell trait), whereas with the normal severe anaemia both parents show sicklaemia (see § 13.5 and Fig. 13.4(*a*)). Further investigation showed that the anaemic children had sickle cell haemoglobin (*S*) and also a new kind of haemoglobin (*C*) which differed

electrophoretically from normal haemoglobin (*A*) and from haemoglobin *S*. Moreover, the non-sickling parent in both families was found to have haemoglobin *C* as well as *A*. The families and their haemoglobins are shown in Fig. 13.4(*b*) and (*c*). It seemed likely that the *C* and *S* haemoglobins were inherited as a pair of alleles since each is recessive to normal and yet the individuals with both abnormalities (*CS*) are anaemic. Hunt and Ingram (1958) found that haemoglobin *C*, like haemoglobin *S*, had an amino-acid substitution in the β-chains of the molecule. Indeed, rather surprisingly, the substitutions were at identical positions, with the glutamic acid at this position in normal adult haemoglobin (*A*) replaced by valine in *S* and by lysine in *C*.

Itano and Robinson (1960) found that an abnormality in the α-chains appeared to be determined by a different genetic locus, which was probably not closely linked to the β-chain gene. The evidence for this came from study of the haemoglobin of relatives of an individual who was heterozygous for the α-chain defect and for haemoglobin *S*. Itano and Robinson (1959) had found that when another kind of haemoglobin called *I*, which is abnormal in the α-chain of the molecule, is mixed with either haemoglobin *S* or haemoglobin *C* (both abnormal in the β-chain), two new kinds of haemoglobin could be produced as well as the original forms. At low pH the α sub-unit of the molecule separates from the β sub-unit, and complete molecules are regenerated when the solution is neutralized. The new kinds of haemoglobin formed in this way consisted of normal haemoglobin *A* and a doubly abnormal molecule combining the defects of both the original types. Baglioni and Ingram (1961) described a comparable situation *in vivo*—a person with four kinds of adult haemoglobin in her blood. These were the normal haemoglobin *A*; haemoglobin *G*, with a lysine residue in place of asparagine at a particular point in each α-chain; haemoglobin *C*, with lysine in place of glutamic acid at a particular point in each β-chain; and haemoglobin *X*, which was found to have both the *G* and *C* substitutions. These four kinds of haemoglobin occurred with approximately equal frequency, suggesting that the mechanism for association of α- and β-chains to form complete molecules did not discriminate between the two kinds of α- or the two kinds of β-chain.

It is evident from these observations that the α- and β-chains are determined by different genes, are synthesized separately, and are then assembled to give haemoglobin by the random association of α- and β-sub-units. In individuals heterozygous for a defect in the α-chain, the two α-chains of each molecule always appear to be alike, and similarly with the β-chain. This is in contrast to the situation described in § 13.6, where association of mutant forms of normally identical sub-units appears to be responsible for the phenomenon of allelic complementation.

Gross (1962) has suggested that two genes in *Neurospora crassa* concerned with the synthesis of leucine, namely *leu*-2 in linkage group IV and *leu*-3 in linkage group I, may be responsible for specifying the amino-acid sequence in two different polypeptide chains which form part of a single enzyme molecule. If the one cistron:one polypeptide hypothesis is found to apply

generally, this will mean that the earlier idea of one gene specifying each enzyme will be true only for enzymes containing one kind of polypeptide.

It appears that the number of cistrons determining a particular enzyme may differ in different organisms. Tryptophan synthetase, the enzyme which catalyses the condensation of indole and serine to give tryptophan (see Fig. 10.1(*b*)), appears to be a double structure, one part (*A*) of the protein molecule having a site which combines with an indole compound (indoleglycerol phosphate), and the other part (*B*) a site which combines with a serine compound (serine + pyridoxal phosphate). In *Escherichia coli*, the *A* and *B* components are specified by different cistrons (Crawford and Yanofsky, 1958)(Fig. 15.1), but in *N. crassa* one cistron (*tryp*-3) determines both activities, which appear to be contained in different parts of a single polypeptide chain (Bonner, Suyama and DeMoss, 1960).

Another example of a bifunctional protein is provided by the enzymes responsible for steps 7 and 9 in the synthesis of histidine in *Salmonella typhimurium* (see Fig. 15.1(*b*)). Both of these steps appear to be catalysed by the same protein. Loper (1961) found that mutants lacking the specific dehydrase catalysing step no. 7 were due to mutation in any part of the gene that specifies the protein, but only those mutants due to mutation in one particular segment also lacked the phosphatase activity of step no. 9. Loper suggested that this protein might be active as the phosphatase under certain conditions, and in a different state of polymerisation would be capable of the dehydrase activity.

The possibility is raised in § 14.15 that the one gene:one polypeptide theory is more correctly stated as one gene:one family of related polypeptides.

§ 13.8 Evidence for co-linearity of gene and polypeptide

Case and Giles (1960) studied the complementation in all pairwise combinations of 75 mutants of *Neurospora crassa* at the *pan*-2 locus. These mutants required pantothenic acid for growth. Of the 75 mutants, 23 showed ability to complement in one or more combinations, while the remaining 52 failed to complement in any combination. The complementation map of the mutants (which are numbered) is shown in Fig. 13.5(*a*). All the complementing mutants and 13 of the 52 non-complementing mutants were mapped genetically. This was done by crossing one with another, selecting for recombinants, and observing the frequency of recombination and the behaviour of outside marker genes. The sequence of the mutant sites on the linkage map so obtained is shown in Fig. 13.5(*b*). No attempt has been made to indicate the recombination frequencies, and the sites are shown uniformly spaced. On this genetic map, the complementing mutants are shown above the line, and the sample of non-complementing mutants below the line.

Comparison of the complementation and genetic maps shows a distinct tendency for the sequence of sites on the two maps to correspond. There are some exceptions to this correspondence, but on the whole the relationship is clear. This is what would be expected if complementation is due to non-overlapping defects in otherwise identical polypeptide chains, and if the site of

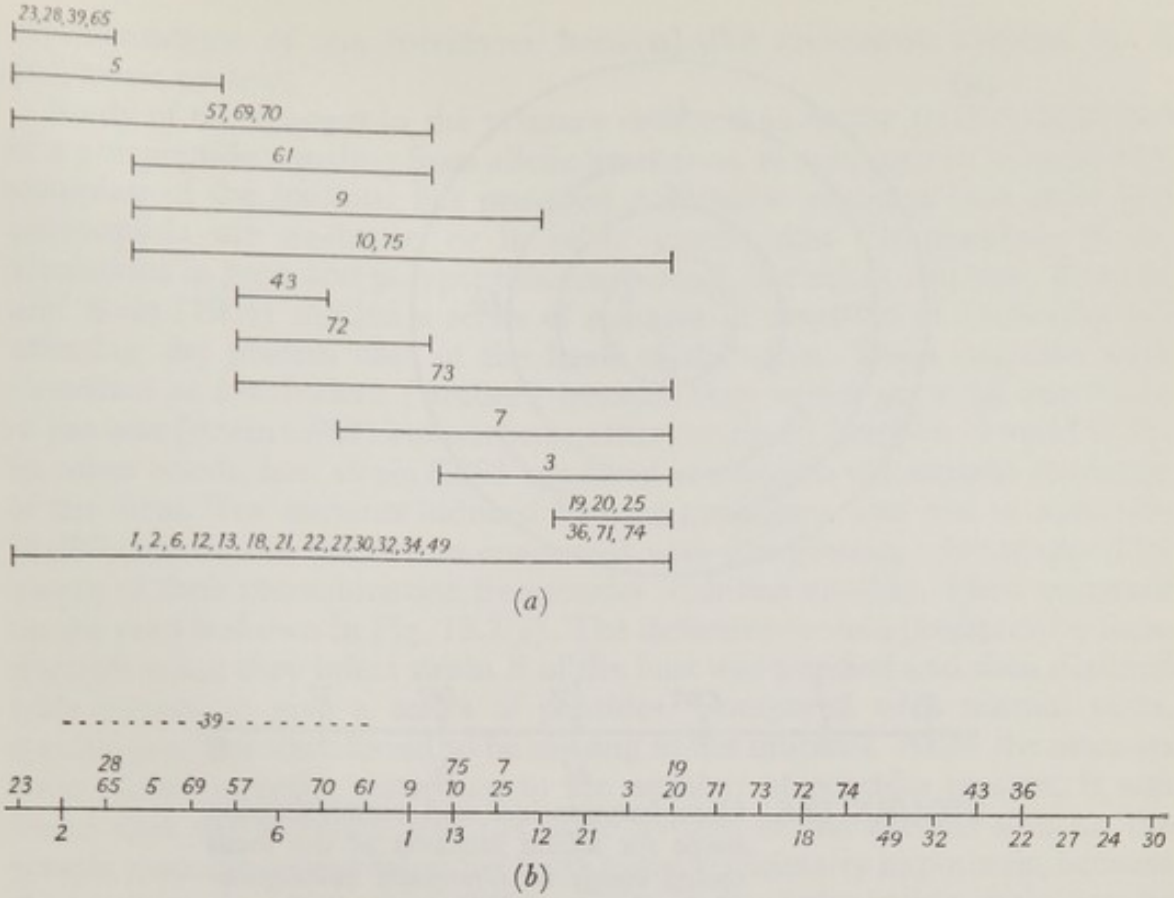


FIGURE 13.5 Data of Case and Giles (1960) for mutants at the pantothenic acid 2 (*pan*-2) locus in *Neurospora crassa*. The numbers specify the mutants.
(*a*) Complementation map.
(*b*) Recombination map. No attempt has been made to indicate recombination frequencies and the mutants are shown uniformly spaced. Complementing mutants are numbered above the line and non-complementing below. A further 39 non-complementing mutants (comparable to the 13 on the bottom line of (*a*)) were not mapped genetically.

modification of the polypeptide chain corresponds to the site of mutation in the gene. Such a relationship between sites in DNA and in the corresponding protein is what would be expected on the Dounce-Gamow hypothesis that nucleotide sequence determines amino-acid sequence. The exceptions to correspondence between genetic map and complementation map would be accounted for if the gene determines the primary structure and the complementation reflects the tertiary structure of the protein, since the pattern of folding to give the tertiary structure is likely to depend on interactions between amino-acid residues in different parts of the polypeptide chain.

Carlson (1961) obtained a circular complementation map for allelic mutants at a locus called *dumpy* in *Drosophila melanogaster* (see Fig. 13.6). In *Neurospora crassa*, circular complementation maps were obtained by Gross (1962) with leucine-requiring mutants (*leu*-2) and by Kapuler and Bernstein (1963) with adenine-requiring mutants (*ad*-8 locus). With *ad*-8, if the genetic map was drawn as a spiral it was found largely to correspond to

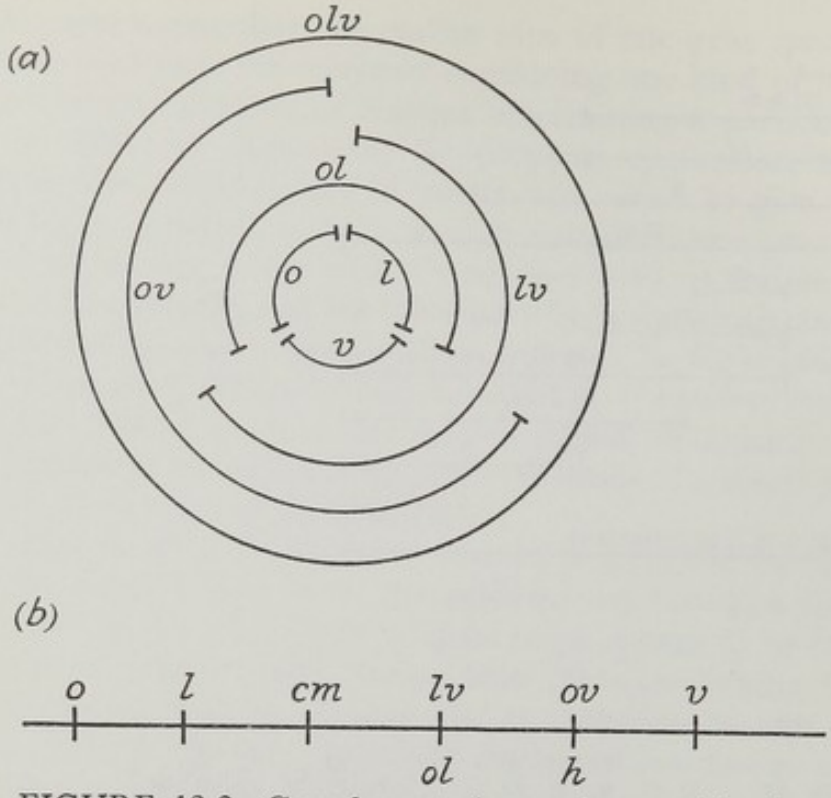


FIGURE 13.6 Complementation and recombination maps for allelic mutants at the locus called *dumpy* in *Drosophila melanogaster* (from Carlson, 1961).
(*a*) Complementation map.
(*b*) Recombination map.
Mutations at this locus show one or more of three recessive effects: an obliquity (*o*) or truncation of the wings; distrubed thoracic bristle patterns known as vortices (*v*); and a lethal effect (*l*). *Comma* (*cm*) is a mutant with weak *o* and *v* effects, and *humpy* (*h*) is a mutant with exaggerated *o* and *v* effects.

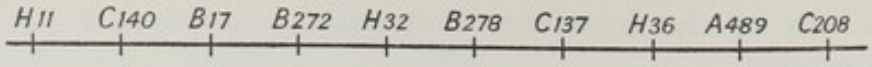
the complementation map. The significance of these circular complementation maps is uncertain. Kapuler and Bernstein suggested that the tertiary configuration of the enzyme may be in the form of a spiral. Crick and Orgel (1964) have concluded that the folding of the polypeptide chain is the key to the relationship between the complementation and genetic maps, but that there is no simple general way of deducing this tertiary structure from them. In a hybrid dimer they believe that the potentially misfolded region of one chain is corrected by the homologous region of the other. Gillie (1966), from an analysis of existing complementation data, has concluded that many loci with linear complementation maps are likely to be found to have non-linear maps when larger samples of mutants are tested. According to Crick and Orgel, each complementing mutant has a derangement of structure near the axis of symmetry of a polymeric protein. Gillie has extended this hypothesis and inferred that complementation maps are

representations of the interfaces between the monomers making up a polymeric protein.

Study of the changes in the primary rather than in the tertiary structure of a polypeptide resulting from allelic mutations, in conjunction with genetic mapping of the mutants has provided conclusive evidence that gene and polypeptide are co-linear, or in other words, that the positions of the alterations in gene and polypeptide correspond. Sarabhai, Stretton, Brenner and Bolle (1964) studied a series of mutants of virus *T*4 of *Escherichia coli* affecting the protein coat of the head of the virus. These mutants were described as ambivalent ('amber') because they would grow on one strain of the host (strain *CR*63) but not on another (strain *B*) (see § 14.17 and § 15.3). In other words, host strain *CR*63 appeared to suppress the mutant character of the virus. Ten mutants induced by base analogues, and due to mutation at different sites in the cistron concerned with this protein, were mapped by means of their recombination frequencies with one another. Their sequence on the map is shown in Fig. 13.7(*a*). The defective protein produced by these mutants when they infect strain *B* of the host was purified and then digested with trypsin to give a series of peptides. Compared with normal virus, specific peptides were found to be missing in the mutants. When the mutants were placed in order according to the number of peptides present, it was found that the sequence was the same as that of the mutant sites on the genetic map. This establishes the truth of the co-linearity hypothesis, because the sequence in which the peptides occur in the polypeptide chain can be inferred from the progressive deficiencies of the mutants (see Fig. 13.7(*b*)). Evidence was obtained that the free amino end of the chain is to the left in the diagram. Since it is known that synthesis of polypeptides begins at the amino end (see § 14.10), the inference is that in the mutants polypeptide synthesis is arrested before completion. Thus, the positions of the mutant sites on the genetic map appear to correspond with the positions where chain synthesis stops in the polypeptide, which is evidently co-linear with the gene. How it comes about that protein synthesis is allowed to go to completion when the virus mutants are grown on host strain *CR*63 instead of strain *B*, is discussed in § 14.17.

Yanofsky *et al.* (1964) and Yanofsky, Drapeau, Guest and Carlton (1967) obtained evidence of the truth of the co-linearity hypothesis by direct comparison of changes in gene and protein. A number of tryptophan-requiring mutants of *E. coli* were obtained by irradiation with ultraviolet light. Those which were unable to convert indoleglycerol phosphate to tryptophan in the presence of serine were selected for study. They were found to be defective in the *A* protein of the enzyme tryptophan synthetase (see § 13.7). This protein is a single polypeptide chain containing 267 amino-acids (Guest *et al.*, 1967). From analysis of the *A* protein produced by the mutants, it was found that each mutant differed from wild-type by a single amino-acid substitution. Details of these substitutions are shown in Fig. 13.8(i), where the letters refer to Fig. 13.2(iii) and identify the amino-acids. The reference numbers of the mutants are given at the top of the diagram. Nearly all the amino-acid substitutions found were within one

(*a*)



(*b*)

H11	
C140	
B17	
B272	Cys
H32	Cys His T7c
B278	Cys His T7c Tyr C12b
C137	Cys His T7c Tyr C12b Try T6
H36	Cys His T7c Tyr C12b Try T6 Pro T2a
A489	Cys His T7c Tyr C12b Try T6 Pro T2a Try T2
C208	Cys His T7c Tyr C12b Try T6 Pro T2a Try T2 Tyr C2
Normal	Cys His T7c Tyr C12b Try T6 Pro T2a Try T2 Tyr C2 His C6

FIGURE 13.7 Data of Sarabhai *et al.* (1964) for 10 mutants affecting the protein coat of the head of virus *T*4 of *Escherichia coli*.
(*a*) Genetic map showing the sequence of the mutant sites. No attempt has been made to indicate recombination frequencies, and the mutants are shown uniformly spaced.
(*b*) The structure of the protein of the coat produced by each mutant, and by the normal virus. The abbreviations refer to the peptides obtained by digestion with trypsin.

quarter of the polypeptide, which is represented by the sequence of 70 letters in the diagram.

In Fig. 13.8(ii) is shown the genetic map of the mutants, which is based primarily on recombination frequencies, but supported by study of crosses with mutants having a segment of the gene deleted. The results show four features of particular significance. First, the genetic map of the mutant sites has the same sequence as the alterations to the polypeptide, thereby demonstrating

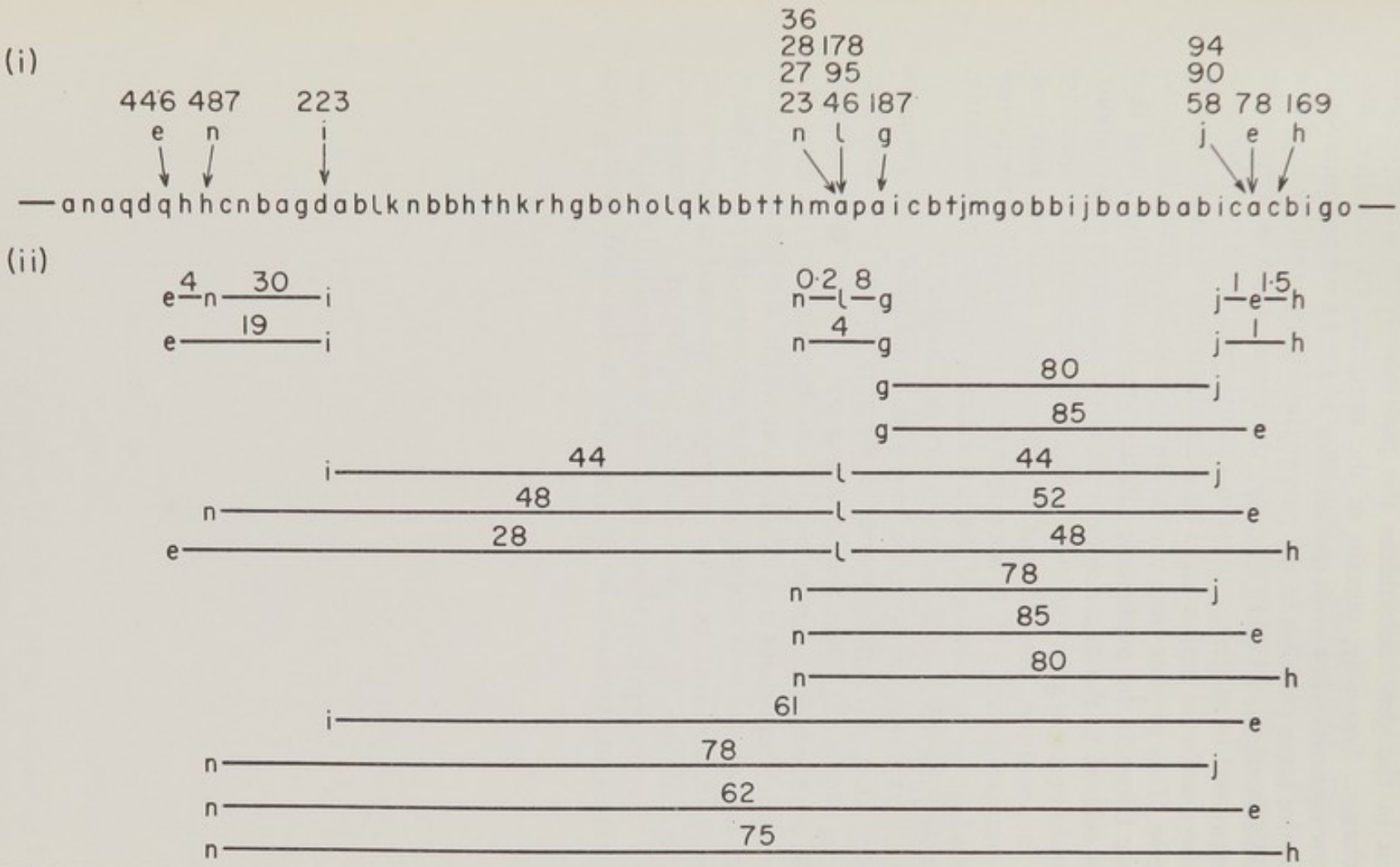


FIGURE 13.8 Data of Yanofsky *et al.* (1964, 1967) for tryptophan-requiring mutants of *Escherichia coli* defective in the *A* protein of the enzyme tryptophan synthetase.

(i) The diagram shows the sequence of amino-acids from no. 169 to no. 238 inclusive in the polypeptide chain of the protein, and the substitutions caused by the numbered mutants. The letters refer to Fig. 13.2(iii). There are 267 amino-acids in the entire polypeptide.

(ii) Genetic map of the mutants. The recombination frequencies are in arbitrary units.

Where there is more than one mutant causing the same amino-acid substitution the recombination frequencies refer to mutants nos. 23, 46 and 58.

the truth of the co-linearity hypothesis. It is evident that the position of a mutation in the gene determines the position of the corresponding amino-acid change. Secondly, certain of the mutants, for example, nos. 23 and 46, show recombination with one another and yet have an amino-acid substitution at the same position in the polypeptide. The significance of this discovery is discussed in § 14.13. Thirdly, the recombination frequencies (which are expressed in arbitrary units in the diagram), are roughly proportional to the number of amino-acids between the points of substitution in the polypeptide. This implies that the recombination frequency is related to the distance between the nucleotide substitutions in the DNA, and that this in turn is related to the distance between the amino-acid substitutions. Fourthly, certain mutants give consistently higher recombination frequencies than others, for example, nos. 23 and 187 compared with 46, and nos. 487 and 223 compared with 446. It is evident that recombination frequency is partly dependent on the nature of the mutations which are recombining. This remarkable conclusion is discussed further in Chapter 16.

All the essential pieces of evidence required to establish the Dounce-Gamow theory that nucleotide sequence specifies amino-acid sequence have now been obtained. When this idea was put forward there were no data available with which to test it, but it now appears that the gene is a linear structure with numerous sites of mutation within the functional unit; that mutations cause changes in amino-acid sequence in specific polypeptides; and moreover, that there is an exact point-by-point relationship between the position of the mutation in the gene and the position of the amino-acid substitution in the polypeptide. How nucleotide sequence in DNA is translated into amino-acid sequence in polypeptides is discussed in the next chapter.

14. *The theory of consecutive non-overlapping triplets of nucleotides as the amino-acid code*

§ 14.1 Introduction

When Dounce (1952) and Gamow (1954) independently put forward the basic idea that nucleotide sequence in nucleic acids might determine amino-acid sequence in polypeptides, they also suggested means by which this translation might be brought about. With four different nucleotides in DNA (having adenine, thymine, guanine, and cytosine respectively as bases), there are 16 possibilities for a sequence of 2 bases, 64 possibilities for a sequence of 3, 256 for a sequence of 4, and so on. Since there are 20 different amino-acids in proteins, it is evident that a minimum of 3 nucleotides is required to specify each. Both Dounce and Gamow therefore suggested that a sequence of 3 consecutive nucleotides was responsible for each amino-acid. Astbury and Bell (1938) had found from X-ray crystallography that the spacing between nucleotides in DNA was approximately the same as the spacing between amino-acid residues in a fully extended polypeptide chain. They considered that this correspondence was likely to be of biological significance, and this led both Dounce and Gamow to suggest that the nucleotide sequence was read at single nucleotide intervals. In other words, if a segment of the nucleic acid is represented by the letters *A B C A C D*, where *A–D* specify the 4 individual nucleotides, the triplet *A B C* would represent one amino-acid, *B C A* the next, *C A C* the third, and so on.

Gamow and Ycas (1955) pointed out that overlapping triplets such as these impose restrictions on the amino-acid sequences that are possible in polypeptides, and yet from an analysis of known sequences which they had made it appeared likely that all possibilities were permitted. They therefore rejected overlapping codes in favour of one in which the number of determining nucleotides exceeded by a factor of 3 the number of amino-acid residues in the synthesized protein, so that neighbouring residues did not share nucleotides. In other words, they favoured a non-overlapping triplet code.

From the range of dipeptides known to occur naturally, Brenner (1957) confirmed that a fully overlapping triplet code was unlikely to be correct. He analysed the published data on amino-acid sequences in polypeptides and found that of the 400 possible sequences in a dipeptide, 239 were already

known. The data referred predominantly to various mammals (*Oryctolagus cuniculus*, *Balaenoptera borealis*, *Equus caballus*, *Bos taurus*, *Ovis aries*, *Sus scrofa*, *Homo sapiens*), but also included one bird (*Gallus domesticus*), one fish (*Salmo* sp.), one flowering plant (*Carica papaya*) and tobacco mosaic virus. If the code was the same in all these organisms, and was fully overlapping and based on trinucleotides, then there could not be more than 256 different dipeptides, since this is the number of ways of arranging a sequence of 4 nucleotides (4^4). Although near this limit, the known number did not exceed it. However, with full overlapping, only 4 different triplets can follow any given one. Thus, after a nucleotide trio represented as *A B C*, the possibilities are *B C A*, *B C B*, *B C C*, and *B C D*. In terms of amino-acid sequence, this would mean that the amino-acid represented by *A B C* could be followed only by four others. In order to allow for the possibility of more than 4 different amino-acids succeeding a particular one in the sequence, it would be necessary to postulate that a second triplet, for example *A B A*, represented the same amino-acid as *A B C*. If all the 20 possible dipeptides which begin with a particular amino-acid are found to occur naturally, a minimum of 5 different triplets would be required to code for this one amino-acid.

Brenner found that there was such diversity in the amino-acids which followed or preceded each particular one, that in total 70 different triplets would be required to code for all 20 amino-acids. This is more than the number possible with a triplet code ($4^3 = 64$). Hence, on the assumption that the organisms from which the amino-acid sequences were obtained had the same code, it was apparent that all fully overlapping codes based on trinucleotides could be ruled out. It could be inferred that if Astbury and Bell's discovery of a similar periodicity in DNA and protein was biologically important, its significance was unlikely to relate to the genetic code.

§ 14.2 The molecular basis of mutation

In order to understand the amino-acid code, it is essential to know in chemical terms the nature of the changes in DNA caused by mutation. Watson and Crick (1953*b*) had suggested that mutation might be due to a base in DNA occasionally occurring in one of its less likely tautomeric forms*, so that at replication the wrong base is inserted at this position in the complementary nucleotide chain. Following the discovery by Dunn and Smith (1954) and Zamenhof and Griboff (1954) that certain base analogues such as 5-bromouracil were incorporated into DNA in place of the specific bases which they resembled chemically (see § 12.4), Litman and Pardee (1956) discovered that 5-bromouracil had a powerful mutagenic effect on virus *T*2 of *Escherichia coli*. This led to a study by Benzer and Freese (1958) of the mutagenic effect of 5-bromouracil on the *r*II cistrons of the *T*4 virus. They made the outstanding discovery that 5-bromouracil did not enhance the spontaneously occurring mutations in a general way, but instead

* Tautomerism is the wandering of a mobile hydrogen atom from one multivalent atom to a neighbouring one within a molecule. Tautomeric forms of a molecule are usually in dynamic equilibrium with one another.

caused mutation at certain sites with characteristic frequencies. Their data relating to the end of the *B* cistron which adjoins the *A* cistron are given in Table 14.1. The pattern of mutations in the *A* cistron and the remainder of the *B* cistron was similar to that shown in the table.

Brenner, Benzer, and Barnett (1958) made similar studies using proflavin as mutagen. Proflavin is known to interact with DNA, but unlike 5-bromouracil does not appear to become incorporated. A finding of very great interest was that proflavin causes mutation at a completely different series of sites from 5-bromouracil (see Table 14.1). The proflavin sites also differed largely from the spontaneously mutating ones. Furthermore, with proflavin there were single occurrences of mutation at many different sites, whereas with 5-bromouracil and also with spontaneous mutation there was more repetition of mutation at certain sites.

Freese and associates studied the *r*II mutations caused by other mutagens such as 2-aminopurine, nitrous acid, and hydroxylamine. The patterns of mutations caused by some of these mutagens on part of the *B* cistron are shown in Table 14.1, which is based on the collected data given by Benzer (1961). Many features of these patterns remain unexplained. Thus, it is not known why certain sites ('hot spots') show extremely high spontaneous mutation frequencies: a possible explanation is discussed in § 16.6. The mechanisms of action of the various mutagens are not understood in chemical terms with certainty. It is clear, however, that their actions are specific for certain sites. This is in direct contrast to the earlier studies on mutation, such as those described in Chapter 9, where the range of kinds of mutations found was independent of the mutagen used. It is evident from the results with *r*II mutants of virus *T*4 that the specificity of mutagen action is at the level of mutant sites within the gene. The early studies on mutation were concerned with the gene as a whole, and at this level of organization the specific effects are lost, just as the totals of the various columns in Table 14.1 would give no indication of the specificity of action of the different mutagens.

Freese (1959) showed that the *r*II mutants produced by the base analogues 2-aminopurine and 5-bromodeoxyuridine (the deoxynucleoside of 5-bromouracil) could be induced to revert to wild-type by the action of these same substances, whereas proflavin-induced mutants and most spontaneous mutants could not. This confirmed the evidence from the differing site patterns that the base analogues on the one hand and proflavin on the other caused mutation in different ways.

Freese suggested that the base analogues caused mutation through mistakes in base pairing, either at the time of incorporation into DNA, or at a subsequent replication. The mechanism was thought to be similar to Watson and Crick's original suggestion, one base temporarily existing in a tautomeric form so that at replication the wrong purine or the wrong pyrimidine would be inserted (see Fig. 14.1(*b*) and (*c*)). Whatever the exact mechanism, the end-result would be that a purine would always be replaced by the other purine, and the corresponding pyrimidine by the other pyrimidine. Freese found that 5-bromouracil-induced mutations were reverted most readily by 2-aminopurine, and *vice versa*, suggesting that these base

TABLE 14.1 The table shows the numbers of mutations that have occurred spontaneously and with various mutagens at 50 different sites within part of the *r*II *B* cistron of *T*4 virus of *Escherichia coli*. (From Benzer, 1961.)

Site	Mutagen							
	Spontaneous	Nitrous Acid	Ethyl Methane Sulphonate	2-amino-purine	2,6-diamino-purine	5-bromo-uracil	5-bromo-deoxy-cytidine	Proflavin
B1/1	1							
2	8							
3	3	1	2	1	1			
4	3							
5	4							
6	1		2		1			
7	2							
8	2							2
9	1							
10	32	14	7	9	1	28	13	
11	2							
12	1							1
13	1							
14		1						
15	1							
16	1							
B2/1	1							
2	2							
3	6							
4	2							
B3/1	5							
2	3							
B4/1	1							
2	5	1						
3								1
4	1							
5	1							
6	1		1					
7	1							
8								2
9	1	8	5	1		3	2	
10	51	4						
11								1
12								1
13				1				
14	2							
15	1		1	1				
16		1						
17	17							1
18	3			4	1			
19	1					2		
20	517	23	7		3	5	5	1
B5/1	1							
B6/1		1						
2	1	3	4			3		
3	1							1
B7/1	1							
2	3		1					
3	2	1						
4	9							

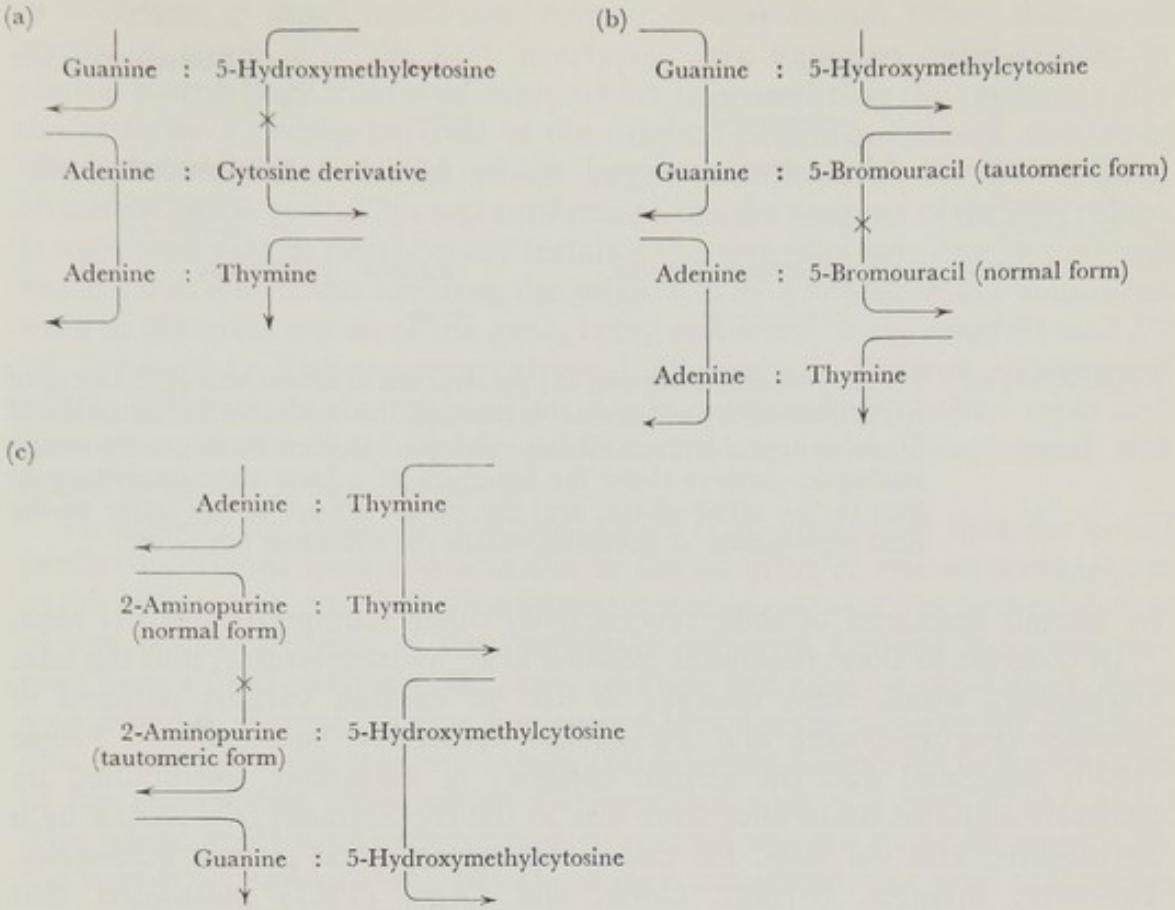


FIGURE 14.1 The hypothetical steps in the production of mutations in *T*4 virus of *Escherichia coli* by (*a*) hydroxylamine, (*b*) 5-bromouracil and (*c*) 2-aminopurine. The steps outlined in (*b*) and (*c*) could also occur in the reverse direction, though this appears to happen less often. Arrows show the insertion of a base complementary to that in the other chain, and the displacement of the latter at the next replication. *X* indicates where the mutagen has acted.

analogues acted predominantly in opposite directions. Later work (Freese *et al.*, 1961) showed that mutants induced by hydroxylamine were similar in properties to those induced by 5-bromouracil. Hydroxylamine is thought to act by altering 5-hydroxymethylcytosine (which replaces cytosine in the DNA of the *T*-even viruses—see § 11.6) so that it pairs with adenine instead of guanine (Fig. 14.1(*a*)). At the next replication, thymine would be inserted opposite the adenine, and the end-result would be that a guanine cytosine-analogue base-pair was replaced by an adenine thymine base-pair. From its behaviour in causing reverse mutation, it was concluded that 5-bromouracil acts predominantly to give the same overall effect as hydroxylamine (Fig. 14.1(*b*)), while 2-aminopurine acts predominantly in the reverse direction, causing the transition from an adenine thymine pair to a guanine cytosine-analogue pair (Fig. 14.1(*c*)). On the other hand, nitrous acid was thought to cause mutation through the oxidative deamination of either adenine or cytosine (and its analogues) (see Fig. 14.2), with the result that transitions from one base-pair to the other could occur more or less equally in both directions. This hypothesis fitted the findings from the induction of mutations

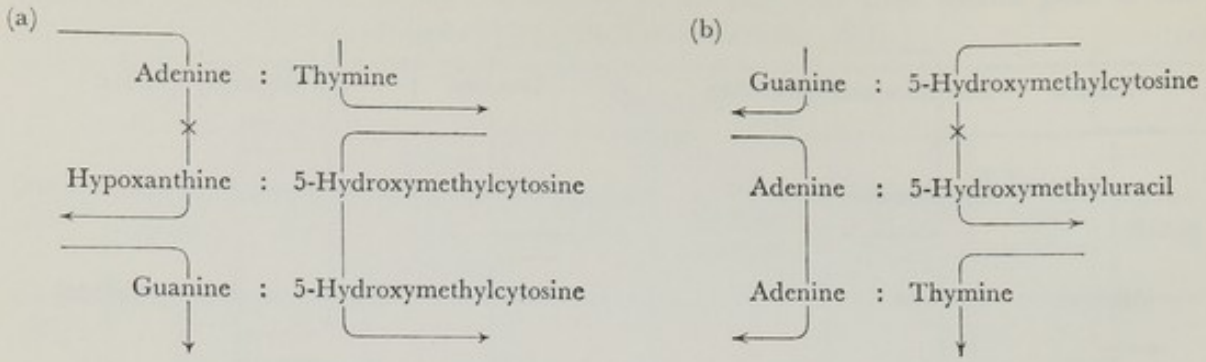


FIGURE 14.2 The hypothetical steps in the production of mutations in *T*4 virus of *Escherichia coli* by nitrous acid, through the oxidative deamination of (*a*) adenine to hypoxanthine, and (*b*) cytosine analogue to uracil analogue. Arrows show the insertion of a base complementary to that in the other chain, and the displacement of the latter at the next replication. *X* indicates where the mutagen has acted.

by nitrous acid and of their reversion by other mutagens, and *vice versa*.

In contrast to these mutagens (nitrous acid, hydroxylamine, and the base analogues), which were thought to act by causing various patterns of *transition* (purine–purine and pyrimidine–pyrimidine substitution), Freese (1959) suggested that the second category of mutations, exemplified by proflavin-induced mutations, were due to the replacement of a purine by a pyrimidine, and *vice versa*. He called this hypothetical process *transversion*. However, Brenner, Barnett, Crick, and Orgel (1961) postulated that proflavin causes mutation by the insertion or deletion of one or more base-pairs in the DNA, and not by transversion as Freese had suggested. Part of their evidence for this was that proflavin-induced mutants appeared completely to lack the normal activity of the gene, whereas mutants produced by other mutagens were often 'leaky', that is, showed some growth on strain *K* of the host (see § 13.4). With many virus characters, proflavin-induced mutants were not found, presumably because loss of the function of the gene controlling them was lethal. This difference in character of proflavin mutants, together with their unique site pattern and the inability of base analogues to cause reversion, suggested that proflavin modified the gene in a fundamentally different way from the other mutagens. That this alteration to the gene was the addition or subtraction of base-pairs was confirmed by the experiments described in the next section.

§ 14.3 Evidence that the code is read in triplets from a fixed origin

Crick, Barnett, Brenner, and Watts-Tobin (1961) performed some ingenious experiments which appear not only to establish the nature of proflavin mutants, but also provide fundamental information about the genetic code. A single *r*II mutant of virus *T*4 of *Escherichia coli* induced by proflavin and situated at a site in segment *B*1 (cf. Table 14.1) formed the starting-point. As indicated in § 13.4, *r*II mutants are unable to grow on strain *K* of the host, thus allowing selection for strains which have reverted

to wild-type. A small number of revertants were found. When these were grown on strain *B* of the host, nearly all were found to differ slightly in plaque morphology from wild-type, which suggested that the revertants did not arise by a precise reversal of the original proflavin-induced mutation, but by some other change which largely compensated for the original alteration to the gene. This was confirmed from the progeny of the revertants. It appeared that in nearly every instance the reversion was due to a second mutation at a different site from the original one. The suppressor mutations were in the same region of the gene, being restricted to segments *B*1 and *B*2 (cf. Table 14.1). This represents about $\frac{1}{10}$ of the whole *B* cistron, only part of which is shown in the table. Each suppressor mutation, when separated through recombination from the proflavin mutant which it suppressed, was found to be an *r*II mutant itself.

To account for these observations, Crick *et al.* postulated that the initial proflavin-induced mutation was due to the addition of one nucleotide-pair at the mutant site, and that the suppressor mutations were due to the deletion of one nucleotide-pair. If the nucleotide sequence forming the gene was read from a fixed starting-point, such addition and subtraction of nucleotides would lead to misreading over the interval between the two sites but nowhere else. In consequence, abnormal amino-acids would be inserted in the segment of the polypeptide chain which corresponded with this part of the gene. This would explain why the suppressed mutant was not quite identical with the wild-type, and also why the site of each suppressor was near that of the original mutant. If the two sites were far apart, the polypeptide chain would be expected to be so abnormal that the phenotype would be mutant, and hence not found when selecting for revertants which would grow on strain *K*.

According to this hypothesis, the original proflavin mutation may be called a plus mutant (Fig. 14.3(*c*)), and the suppressors of it may be called minus mutants (Fig. 14.3(*b*)–(*d*)). By repeating the original procedure, suppressors of the minus mutants were obtained (Fig. 14.3(*h*) and (*i*)). They were found to be due to mutation at different sites near the site of the mutant they suppressed, just like the first group of suppressors. According to the hypothesis, this second family of suppressors should be plus mutants, and this was confirmed experimentally, because two plus mutants brought into the same piece of DNA through recombination had the mutant phenotype like each alone (Fig. 14.3(*j*)), and similarly with two minus mutants (Fig. 14.3(*k*)), but one plus and one minus mutant suppressed one another and gave the wild-type or nearly wild-type phenotype, provided they were not too far apart. By selecting for revertants of these plus mutants, a third family of suppressors was obtained and found to be minus mutants, as expected. Altogether about 80 *r*II mutants were obtained by this successive selection of suppressors, and their sites extended over about $\frac{1}{4}$ of the *B* cistron.

An outstanding discovery was the finding that 3 plus mutants in the same piece of DNA gave a near wild-type phenotype, and similarly with 3 minus mutants (Fig. 14.3(*l*) and (*m*)). On their hypothesis, this implied that the *coding ratio*, that is, the ratio of bases to amino-acids, was 3. The addition or deletion of 3 nucleotides within a short segment of the DNA had restored

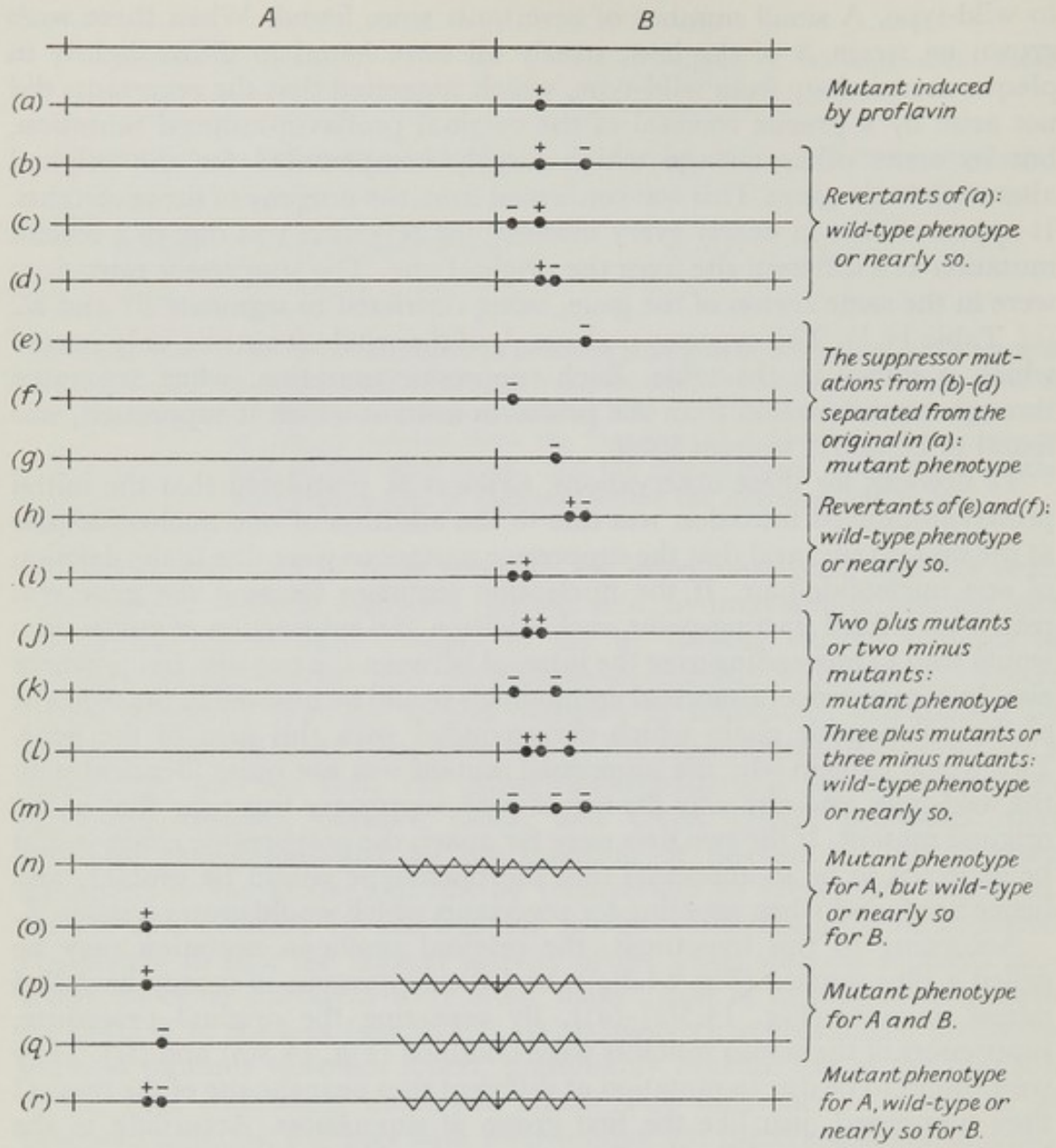


FIGURE 14.3 Results obtained by Crick *et al.* (1961) from study of suppressors of a proflavin-induced *r*II mutant of virus *T*4 of *Escherichia coli*.

the function of the gene, indicating that the nucleotides were read in threes from one end. With the addition or subtraction of 1 or 2 nucleotides the gene is evidently misread to the end, resulting in the mutant phenotype, but with 3 added or removed it is misread only over the interval between the sites. Thus, their results suggested that a triplet of nucleotides was the coding unit or *codon* for an amino-acid.

Further evidence that the gene is read from a fixed starting-point was obtained from the use of a deletion known to cover parts of both *A* and *B* cistrons in the region where they join. The loss of this segment destroys the function of the *A* cistron, but not that of the *B*: it covers the part of the *B* cistron where the mutants used in the present study are situated. This is presumed to be a non-essential part of the *B* gene (Fig. 14.3(*n*)). A mutant in the *A* cistron which reverts freely with proflavin and is therefore presumed

to be an addition (or deletion) of a nucleotide pair, destroys the function of the *A* gene without affecting the *B*, as might be expected (Fig. 14.3(*o*)). However, when this mutant and the deletion are combined in the same piece of DNA, it was found that the function of the *B* cistron was also destroyed (Fig. 14.3(*p*)). It was presumed that with the loss of the junction between *A* and *B* cistrons, both were being read as one, with the result that putting the reading out-of-phase in the *A* cistron affected the *B* as well. This was confirmed by the finding that a suppressor of the *A* cistron mutant restored the *B* cistron function (Fig. 14.3(*r*)). It thus appears that the gene (or nucleotide sequence derived from it) is read in trios of nucleotides starting from a fixed origin at one end.

It was assumed that the initial proflavin-induced mutant in this series of experiments had an addition of one nucleotide-pair at the mutant site in the *B* cistron. However, this mutant might equally well have been a deletion of one nucleotide-pair. This would not affect the argument except to interchange plus and minus throughout. Crick and Brenner (1967) have evidence that the plus and minus signs, as originally used, may be correct. The initial mutant, and the series of suppressors obtained from it, might represent additions or deletions of 2 nucleotide-pairs instead of only 1. Although improbable, this remains a possibility, with its corollary that the coding ratio may be 6. However, independent evidence that the coding ratio is 3 has since been obtained (see § 14.11 and § 14.12).

Clear evidence for the validity of the arguments of Crick *et al.* (1961) has since been obtained (see § 14.13).

§ 14.4 RNA

In attempting to discover how the nucleotide sequence in DNA is translated into amino-acid sequence in protein, it is essential to know whether the translation is direct, or whether other substances intervene in the process. The work of Brachet, Caspersson and many others (see Brachet, 1957) established that ribonucleic acid (RNA) participates in protein synthesis. One of the clearest demonstrations of this was when Brachet showed that treatment of cells with ribonuclease, which catalyses the breakdown of RNA, stops protein synthesis. Whereas DNA is largely confined to the nucleus, RNA occurs predominantly in the cytoplasm, although it is found in the nucleolus as well as in variable quantity in association with the chromosomes. Brachet demonstrated with the green alga *Acetabularia* (cf. § 5.3) that protein synthesis could continue in the cytoplasm in the absence of the nucleus. In many tissues a correlation has been shown between the amount of RNA present and the rate of protein synthesis. From observations such as these the idea developed that RNA provides a link between gene and protein. More specifically, it was suggested that information in the form of base sequence in DNA was transferred to RNA and thence to amino-acid sequence in proteins. The size of the RNA molecule (see below) is such that it could readily pass through the 40 mμ wide pores in the nuclear membrane.

The first clear evidence that RNA has the capacity to carry information

about protein structure came from work on tobacco mosaic virus. This virus contains no DNA. It is composed of RNA surrounded by a hollow cylinder of protein measuring 300 mμ in length and 15 mμ in external diameter. Gierer and Schramm (1956) separated the RNA and the protein and showed that the RNA by itself is infective, although to a much lesser degree (0·1%) compared with the intact virus. Fraenkel-Conrat (1956) mixed the RNA of one strain and the protein of another, and produced

TABLE 14.2 The table shows the results obtained by Fraenkel-Conrat and Singer (1957) from study of two strains of tobacco mosaic virus, and of reciprocal chimaeras of their RNA and protein.

N = Normal *Nicotiana* strain of virus
P = *Plantago* strain of virus, sometimes called ribgrass virus.

The first and last columns of figures give the numbers of the various amino-acid residues in the N and P proteins, according to the data of Tsugita (1962). Both contain a total of 158 amino-acids. The other figures are Fraenkel-Conrat and Singer's values for the percentage weight of each amino-acid in the virus strains. The analyses were not corrected for destruction of acid-sensitive amino-acids during hydrolysis.

Strain of Virus							
		RNA	*N*	*N*	*P*	*P*	
		Protein	*N*	*P*	*N*	*P*	
Serological character			*N*	*P*	*N*	*P*	
Nature of disease produced by progeny			*N*	*N*	*P*	*P*	
Serological character of progeny			*N*	*N*	*P*	*P*	
Amino-acid composition of protein of progeny	*a*	Glycine	6	2·3	2·3	1·8	1·6	4
	b	Alanine	14	6·5	6·9	8·5	8·5	18
	c	Serine	16	9·0	8·8	8·1	8·1	13
	d	Threonine	16	8·9	8·9	7·5	7·2	14
	e	Cysteine	1	Not determined (about 0·6%)				1
	f	Methionine	0	0·0	0·0	2·2	2·0	3
	g	Valine	14	9·6	9·0	6·3	5·9	10
	h	Leucine	12	14·2	14·3	12·2	12·2	11
	i	Isoleucine	9					8
	j	Aspartic acid	18	13·8	14·2	14·8	15·0	17
	k	Asparagine						
	l	Glutamic acid	16	12·4	12·1	17·3	16·4	22
	m	Glutamine						
	n	Arginine	11	9·5	9·7	8·5	8·9	11
	o	Lysine	2	1·9	1·8	2·3	2·4	2
	p	Phenylalanine	8	7·2	7·1	5·4	5·3	6
	q	Tyrosine	4	4·1	4·3	6·2	6·3	7
	r	Histidine	0	0·0	0·0	0·7	0·7	1
	s	Tryptophan	3	2·8	2·6	2·2	2·2	2
	t	Proline	8	5·0	5·1	5·1	5·0	8

several very active chimaeras. The serological characters of these mixed virus preparations were those of the virus supplying the protein, but all the characters of the progeny were those of the virus supplying the RNA.

Fraenkel-Conrat and Singer (1957) investigated this remarkable discovery in more detail. One of the best examples concerned chimaeras derived from the normal virus and a strain originally isolated from *Plantago lanceolata*. On a Turkish variety of *Nicotiana tabacum*, the normal virus produces mottling of the leaves while the *Plantago* strain produces a distinctive ring pattern of diseased cells. Furthermore, the two strains differ appreciably in the composition of their protein, and in antigenic specificity. The protein of the

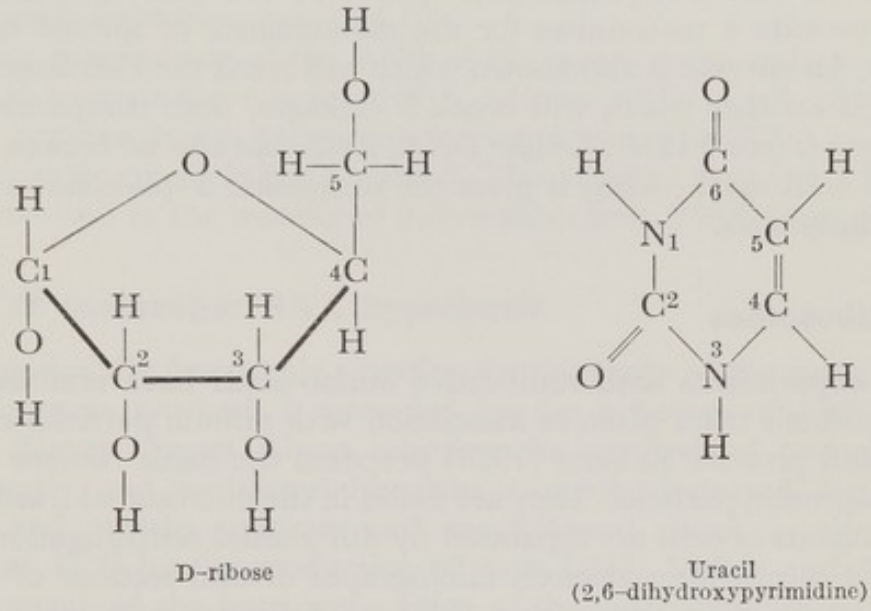


FIGURE 14.4 The components of RNA which differ from those of DNA (D-ribose in place of 2-deoxy-D-ribose, and uracil in place of thymine; cf. Fig. 11.3).

Plantago strain (*P*) contains methionine and histidine which are absent from the normal *Nicotiana* strain (*N*), and there are differences in frequency of nearly all the amino-acids (see Table 14.2). Indeed, the *Plantago* strain differs so much that it is regarded by some authors as a distinct species and is called ribgrass virus. Fraenkel-Conrat and Singer prepared reciprocal chimaeras, that is, RNA of the *N* strain with protein of the *P* strain, and *vice versa*. They found that the progeny of each had protein closely corresponding in amino-acid composition to that of the parent from which the RNA was derived (see Table 14.2). It appeared that the protein part of the virus made no contribution to the progeny, and that the specificity of the virus protein was determined solely by the RNA.

In view of this clear evidence that RNA can carry genetic information about protein structure, it is not surprising that the structure of RNA appears to be quite similar to that of DNA. The essential differences are that the sugar is D-ribose (Fig. 14.4(*a*)) instead of 2-deoxy-D-ribose, and one of the pyrimidines is uracil (Fig. 14.4(*b*)) in place of the thymine (5-methyluracil) of DNA. The other three bases (adenine, guanine and cytosine) are the same.

The nucleotides are polymerized by 3′,5′-phosphodiester linkages as in DNA to form polynucleotide chains. These nucleotide chains are thought normally to occur singly, unlike DNA. However, there may be pairing of complementary bases between different parts of the same chain to give double-helical regions.

Grunberg-Manago and Ochoa (1955) isolated from *Azotobacter vinelandii* an enzyme, polynucleotide phosphorylase, which would bring about RNA synthesis *in vitro* from ribonucleoside 5′-diphosphates with release of orthophosphate. The reaction requires magnesium ions, but no primer is needed, and it is not necessary to have all 4 ribonucleoside 5′-diphosphates present. In view of the lack of requirement for primer, it is evident that this enzyme does not provide a mechanism for the maintenance of specific nucleotide sequences. An enzyme is also known which will break the 3′-linkages of RNA chains, and another which will break 5′-linkages, both comparable to the DNA enzymes (see § 12.4). Unlike DNA, RNA can also be broken down by hydrolysis with alkali, when it gives the nucleoside 3′-phosphates and also some 2′-phosphates.

§ 14.5 Ribosomes

Tracer experiments with radioactive amino-acids have established that protein synthesis takes place in association with minute particles composed of RNA and protein. Roberts (1958) proposed the name *ribosome* for these ribonucleoprotein particles. They are found in the microsomal fraction when the components of cells are separated by differential centrifugation. Palade (1955) had shown from electron micrographs of thin sections of about 40 different mammalian tissues that these particles occur in large numbers in all the cells. Many were associated with the membrane system of the endoplasmic reticulum (cf. Plate 5(*a*)). In bacteria, however, where there may be several thousand ribosomes per cell, they are apparently not associated with membranes.

Ribosomes seem to be a fundamental constituent of all cells and to be very similar in all organisms. They are more or less spherical in shape and measure approximately 20 mμ across. Ribosomes from bacteria are slightly smaller, however, than those of chromosomal organisms: their sedimentation coefficients are about 70 and 80 Svedberg units, respectively. Moreover, bacterial ribosomes contain about 50 % protein and 50 % RNA, while those of chromosomal organisms contain about 60 % protein. The ribosomes found in the chloroplasts and mitochondria of chromosomal organisms, however, are of bacterial type (see § 18.11).

At low concentrations of magnesium ions, ribosomes dissociate into two sub-units. In bacterial ribosomes these have sedimentation coefficients of about 50*S* and 30*S*. The 50*S* sub-unit yields RNA components of 23*S* and 5*S*, and the 30*S* sub-unit gives a 16*S* RNA component. The 5*S* component has been studied by Brownlee *et al.* (1967) who determined the entire sequence of the 120 nucleotides in its RNA, using techniques described in § 14.11. More than half the nucleotides are unpaired, but the two ends of

the molecule have complementary sequences of bases and there are two other paired regions also.

The proteins of the 30*S* sub-unit have been studied by Moore *et al.* (1968). Thirteen proteins were separated by electrophoresis and ion exchange chromatography and purified. Each was then characterized by determining its amino-acid composition, tryptic fingerprint and molecular weight, and all were found to be different. There was one copy of each per 30*S* sub-unit. Moore *et al.* estimated that there may be about 20 proteins altogether in the 30*S* sub-unit and about 40 in the 50*S* sub-unit. They suggested that each fulfils a specific function in relation to the mechanism of protein synthesis. It is already known that a protein in the 50*S* sub-unit is an enzyme involved in polypeptide formation (see § 14.10).

The base composition of the RNA of ribosomes seems to be remarkably uniform: in bacteria there is about 26 % adenine, 20 % uracil, 32 % guanine and 22 % cytosine (molar proportions), irrespective of the DNA composition of the species from which it is obtained. This suggests that this RNA is not directly involved in the transfer of information from DNA to protein.

§ 14.6 The messenger RNA hypothesis

An exceptionally favourable situation for the study of the part played by RNA in protein synthesis is provided by the infection of *Escherichia coli* with the *T*-even viruses. Upon infection the synthesis of bacterial DNA stops abruptly, and the bacterial nucleus is rapidly destroyed, but synthesis of RNA and protein continue and are followed about 7 minutes after infection at 37°C by the production of virus DNA. Volkin and Astrachan (1957) determined the base composition of the newly-synthesized RNA by transferring infected bacteria to a medium containing labelled phosphorus (^{32}P), hydrolysing the RNA shortly afterwards, separating the 4 classes of nucleotides, and measuring their radioactivity. They found that the newly synthesized RNA was similar in base composition to the virus DNA (apart from having uracil in place of thymine, and cytosine in place of 5-hydroxymethylcytosine). This composition was different from that of the bacterial DNA, and from the overall composition of the RNA, 80 % of which is in the ribosomes (see Table 14.3). These observations point to the newly synthesized RNA as a carrier of information from DNA to protein. It was notable also that this RNA showed a high turnover rate, with no net synthesis.

Hall and Spiegelman (1961) showed that the RNA synthesized after infection of *E. coli* by *T*2 virus could form hybrid molecules with the DNA of the virus. Owing to its difference in base composition, the newly-synthesized virus-specific RNA can be separated from the bacterial RNA by density-gradient centrifuging in sucrose solution. Hall and Spiegelman then mixed the virus-specific RNA with the virus DNA. The DNA had previously been heated to 95°C for 15 minutes, and then quickly placed in an ice-bath. Marmur and Lane (1960) had shown that such heating of DNA causes the complementary nucleotide chains to separate and that slow cooling allows them to re-associate again in complementary pairs, but quick cooling does

not. The mixture of RNA and single-chain DNA was placed in a water-bath at 65°C and allowed to cool very slowly, taking 30 hours to reach 26°C. Hall and Spiegelman found that hybrid DNA-RNA molecules were thereby formed. Their evidence for this was based on radioactive isotope labelling using ^{32}P in phosphate for the culture which provided the RNA, and ^{3}H in thymidine for that which provided the DNA. In a control experiment, the labelled RNA and DNA were mixed, but not heated, and then centrifuged

TABLE 14.3 Base composition of the DNA and RNA of *Escherichia coli* and its *T*-even viruses expressed in molar percentages.

Base	*Escherichia coli*		*T*-even Viruses		
				RNA specific to Virus	
	DNA	Total RNA	DNA	Volkin & Astrachan 1957	Bautz & Hall 1962
Adenine	25	23	32	31	30
Thymine (DNA) or Uracil (RNA)	25	23	32	29	33
Guanine	25	31	18	22	21
5-Hydroxymethylcytosine (virus DNA) or Cytosine (RNA and bacterial DNA)	25	23	18	18	16

in caesium chloride solution for 5 days to give a density gradient. The contents of the centrifuge tube were then removed drop by drop in sequence and assayed for ^{32}P and ^{3}H. The β-particles emitted by these isotopes have different energies and so can be distinguished. It was found that the ^{32}P and ^{3}H were concentrated at different positions in the tube, as expected since RNA is denser than DNA. By contrast, after heating and slow-cooling the DNA-RNA mixture, the ^{32}P showed a bimodal distribution, the new peak partly overlapping the ^{3}H peak. It was inferred that the heating and slow cooling had allowed hybrid DNA-RNA molecules to form, and that these had a density close to that of DNA. The hybrid complex evidently contained considerably more DNA than RNA, presumably because the RNA was only a short segment.

Confirmation that hybrid molecule formation depends on complementary nucleotide sequences in the DNA and RNA was obtained by slow-cooling

mixtures of the *T*2-specific RNA and previously-heated DNA of the host bacterium, *Escherichia coli*. No hybrid molecules were formed. Likewise there was no hybrid formation with the heated DNA of *Pseudomonas aeruginosa* nor with that of virus *T*5 of *E. coli*, which happens to have the same overall base composition as *T*2. Hall and Spiegelman's results thus provide a clear indication that the RNA formed after *T*2 infection is likely to have been synthesized in association with the virus DNA, since the RNA appears able to form double helices with single chains of *T*2 DNA, but not with other sources of DNA.

Weiss and Nakamoto (1961) and Hurwitz, Furth, Anders, Oritz, and August (1962) have described an RNA polymerase, both from bacteria and mammals, which has many features in common with the DNA polymerase described by Kornberg and associates (see Chapter 12). The RNA polymerase requires all 4 ribonucleoside 5′-triphosphates as substrate, and DNA as primer. Divalent metal ions are also needed, and pyrophosphate ions are liberated. This enzyme, first detected in the nuclei of the liver of *Rattus norvegicus*, has since been found in extracts of several bacteria and *Pisum sativum*. The reaction catalysed would appear to provide a means by which base sequence in DNA is transferred to RNA. Following RNA synthesis *in vitro* with this enzyme using 5′-triphosphates one of which was labelled with ^{32}P, the RNA was broken down again by alkaline hydrolysis into 3′-phosphates (some 2′-phosphates were also formed), and the proportion of radioactive phosphorus associated with the 4 different nucleotides was determined. By labelling each nucleoside 5′-triphosphate in turn with ^{32}P, the relative frequencies of the 16 possible sequences of a pair of nucleotides were estimated (cf. § 12.5). Using the DNA from various organisms in turn as primer, it was found that the base sequence in the synthesized RNA always resembled that of the DNA primer.

Chamberlin and Berg (1962) obtained similar results. That the DNA and RNA should have the same proportions of the 4 bases suggests that each of the complementary nucleotide-chains in the primer DNA was used for the synthesis of RNA chains. This was confirmed by Chamberlin and Berg by using as primer the DNA of virus $\phi X174$ of *E. coli*. This virus normally contains only one particular nucleotide chain of DNA (Sinsheimer 1959) with a molar base composition of 25% adenine, 33% thymine, 24% guanine, and 18% cytosine. The RNA synthesized when the $\phi X174$ single-chain DNA was used as primer had the complementary composition (34% adenine, 24% uracil, 19% guanine, and 23% cytosine). When DNA of this virus with normal complementary pairs of chains, obtained *in vitro* with the DNA polymerase, was used as primer for the RNA polymerase reaction, the resulting RNA had a base composition of 29% adenine, 29% uracil, 21% guanine, and 21% cytosine, which is identical with the DNA composition (except for uracil in place of thymine). This indicates that both DNA nucleotide chains were used for RNA synthesis. However, it cannot be inferred from this that RNA synthesis *in vivo* will necessarily occur in relation to both DNA chains. Indeed, evidence is given in § 14.7 which shows that RNA synthesis *in vivo* occurs in relation to one particular DNA chain only.

The idea that the short-lived RNA formed after virus infection constitutes a messenger between DNA and protein obtained strong support from some ingenious experiments by Brenner, Jacob, and Meselson (1961). *Escherichia coli* was grown for several generations in a culture medium containing the heavy isotopes ^{15}N and ^{13}C, so that all the nitrogen and carbon in the ribosomes would be of these heavy atoms. The bacteria were then infected with virus *T*4, and transferred immediately to a medium containing normal nitrogen (^{14}N) and carbon (^{12}C). In one experiment the bacteria were fed with phosphate containing radioactive phosphorus (^{32}P) from the second to the seventh minute after transfer, and in another experiment they were given sulphate containing radioactive sulphur (^{35}S) for the first 2 minutes of virus multiplication. The cells were subsequently disrupted and the ribosomes purified and centrifuged in caesium chloride solution to give a density gradient (cf. § 12.3). Control experiments showed that this treatment would separate any newly-synthesized ribosomes containing ^{14}N and ^{12}C from the old heavy ribosomes containing ^{15}N and ^{13}C. It was found that no wholly new ribosomes were synthesized after the virus infection, and moreover, the newly-synthesized RNA indicated by ^{32}P, and the newly-synthesized protein indicated by ^{35}S, were found to be associated with the heavy band of ribosomes in the caesium chloride gradient. It was evident that at the time of cell disruption virus protein was being synthesized in old bacterial ribosomes under the influence of an unstable RNA fraction which was presumed to act as a messenger from the virus DNA. It appeared that the ribosomes were non-specialized structures which synthesize at a given time the protein dictated by the messenger they happen to contain. The function of the ribosomal RNA was not revealed by these experiments and remains unknown.

Nirenberg and Matthaei (1961) obtained evidence that the messenger RNA must be in the form of a single chain in order to function. In a cell-free system derived from *Escherichia coli*, a synthetic polyribonucleotide containing uracil but no other bases, and hence necessarily in the form of single chains, was found to lead to a specific type of protein synthesis (see § 14.12) whereas when the uracil chains were paired with adenine chains, no such synthesis occurred.

Evidence pointing to the existence of messenger RNA in chromosomal organisms was obtained by Ycas and Vincent (1960). They allowed *Saccharomyces cerevisiae* to grow in the presence of orthophosphate containing radioactive phosphorus (^{32}P), and found that the newly-synthesized RNA into which it was incorporated had a molar base composition of 32% adenine, 29% uracil, 19% guanine, and 20% cytosine. This is similar to the DNA composition: 31·5% adenine, 33% thymine, 18·5% guanine, and 17% cytosine, and quite different from the overall RNA composition (chiefly ribosomes) of 25% adenine, 28% uracil, 23% guanine, and 24% cytosine. A number of authors have since obtained similar evidence from mammalian tissues. These observations suggest that messenger RNA is of general occurrence (see also § 18.7).

§ 14.7 Evidence that one particular nucleotide chain of DNA is transcribed

Bautz and Hall (1962) isolated virus *T*4 messenger RNA from recently infected cells of *Escherichia coli* by absorbing the RNA at 55°C in a cellulose column containing virus *T*4 DNA in the single-chain condition following heating. The RNA was subsequently separated from the DNA by heating to 65°C, and then purified, and hydrolysed to find its base composition. The results obtained are shown in Table 14.3 and are in reasonable agreement with those of Volkin and Astrachan discussed in § 14.6 and obtained by a different method. In view of this agreement, Bautz and Hall attached real significance to a feature of their data of great interest, namely, that the total content of guanine and cytosine in the messenger RNA (37%) is approximately equal to the corresponding total for the virus DNA (36%), but the individual bases occur unequally in the RNA (21% guanine, 16% cytosine). These observations would be accounted for if the messenger RNA is always synthesized as the complement of one particular nucleotide-chain of the DNA, and if this chain contains 21% cytosine-analogue and 16% guanine. A similar argument can be applied to the data for the other pair of bases.

Evidence that only one specific DNA chain appears to be made use of when a gene function was also obtained by Champe and Benzer (1962) from study of the effect of 5-fluorouracil on the expression of certain *r*II mutants of *T*4 virus. This base analogue is thought to be incorporated into RNA in place of uracil, but then to show hydrogen bonding similar to cytosine. In consequence, an *r*II mutant which has uracil in place of cytosine at one point in its messenger RNA will lose its mutant character and show the normal phenotype if 5-fluorouracil has taken the place of the uracil. The adenine in the DNA chain complementary to the uracil will be unaffected, so the mutant character will reappear in the progeny. Of 46 *r*II mutants thought from their reversion behaviour with mutagens (see § 14.2) to be due to a transition from a guanine cytosine-analogue base-pair to an adenine thymine pair, 29 were found to give the wild-type phenotype with 5-fluorouracil, while the remaining 17 did not respond. This result would be accounted for if the group of 29 mutants had one orientation of adenine and thymine with respect to the two DNA chains and the group of 17 mutants had the other orientation, and if the messenger RNA was synthesized as the complement of only one specific nucleotide chain of the DNA. The 29 mutants which responded to the uracil analogue were assumed to have adenine in this DNA chain at the mutant site, and hence normally would have uracil in the messenger RNA, while the other 17 were presumed to have thymine in the DNA chain and hence adenine in the messenger RNA.

Further evidence that only one of the two chains of the DNA is transcribed into messenger RNA has been obtained from several sources. In *Diplococcus pneumoniae* the two nucleotide chains of the DNA differ sufficiently in base composition to form separate bands when centrifuged in caesium

chloride solution. Guild and Robison (1963) obtained preparations of the two fractions by this means. Using DNA from a novobiocin-resistant strain of the bacterium, they found that the heavy fraction (richer in the heavier purine, guanine, and the heavier pyrimidine, thymine) required about 45 minutes at 37°C to transform novobiocin-sensitive cells to resistance, whereas the lighter fraction modified the phenotype almost immediately. Since the cell generation time is about 40 minutes at 37°, it was inferred that the heavier chain needs to replicate before effective messenger RNA can be synthesized, whereas the lighter chain does not. It appeared likely that the messenger RNA from the gene for novobiocin-resistance was always complementary to the lighter of the two DNA chains.

Fox and Meselson (1963) obtained virus lambda (λ) of *Escherichia coli* in which the thymine of the DNA was replaced by 5-bromouracil. Following mixed infection of the host with this and with normal λ, those progeny virus which had one chain of the DNA normal and the other containing 5-bromouracil were isolated by density-gradient centrifugation. Virus particles in which both chains of the DNA contain 5-bromouracil are rapidly inactivated by exposure to light. It was found that when the virus particles in which one chain contained 5-bromouracil and the other did not were exposed to light, half the population survived. This would be accounted for if one specific chain of the DNA was essential for the synthesis of messenger RNA which was needed for a function upon which the replication of the DNA was dependent. If this DNA chain happened to be the one containing 5-bromouracil, no messenger would be formed and the virus DNA would be unable to replicate. In the other half of the population of virus particles, the 5-bromouracil would be in the complementary DNA chain, damage to which would not prevent the formation of messenger RNA.

Studies with $\phi X174$ and certain other bacterial viruses in which the two chains of the DNA can be separated, either because of a difference in molecular weight or because all the mature virus contains only one specific chain, have shown that the messenger RNA formed after infection has a base composition similar to one chain of the virus DNA and hence complementary to the other. With virus $\phi X174$, progress has been made in understanding why the formation of messenger RNA *in vivo* appears to be complementary to one DNA chain, while *in vitro* it may be formed complementary to both (see § 14.6). This virus has its DNA in the form of a single molecule with the ends joined to form a closed circle. Hayashi, Hayashi, and Spiegelman (1964) purified chromatographically the DNA of $\phi X174$ obtained during its replication in the host, *E. coli*. Although the mature virus contains only one specific nucleotide chain (see § 14.6), complementary chains are present during replication. It was confirmed both from electron micrographs and from sedimentation patterns that at least 90% of the virus DNA molecules retained their circular form. The RNA synthesized *in vitro* with the RNA polymerase using the circular double-chain $\phi X174$ DNA as primer was found to resemble one of the DNA chains in composition, whereas when the DNA was fragmented by means of a sonic oscillator, the RNA resembled both chains. This was established from the

relative frequencies with which ^{32}P, incorporated into the RNA in guanosine 5′-phosphate, was recovered in the 3′- (and 2′-) phosphates of each of the 4 nucleosides after alkaline hydrolysis, because the relative frequency of the 4 bases on the 3′ side of guanine in one DNA chain happens to be quite different from that in the other chain. Confirmation of the results was obtained by heating and slow cooling the RNA and the single-chain DNA of the mature virus. The RNA synthesized with disrupted double-chain DNA as primer gave rise to hybrid molecules with the single-chain DNA, whereas the RNA formed with the unbroken double-chain DNA did not. The inference from these experiments is that messenger RNA produced from the unbroken two-chain DNA resembles the single DNA chain of the mature virus in composition. It is evident that fragmentation of the DNA causes breakdown of the mechanism which determines that the messenger RNA shall be formed as the complement of one specific DNA chain.

§ 14.8 The direction of transcription (synthesis of messenger RNA)

There are two possible directions for RNA synthesis: the nucleotides might be added (as nucleoside triphosphates with elimination of pyrophosphate) at the 5′-phosphate or at the 3′-hydroxyl end of the chain (Fig. 14.5(*a*) and (*b*)). With the first alternative the triphosphate group at one end of the growing RNA molecule will be that of the last nucleoside to be added and so will be continually replaced as synthesis proceeds, while with the second alternative the initial nucleoside will retain its triphosphate group indefinitely. The direction of synthesis was established by making use of this and other differences between the alternatives.

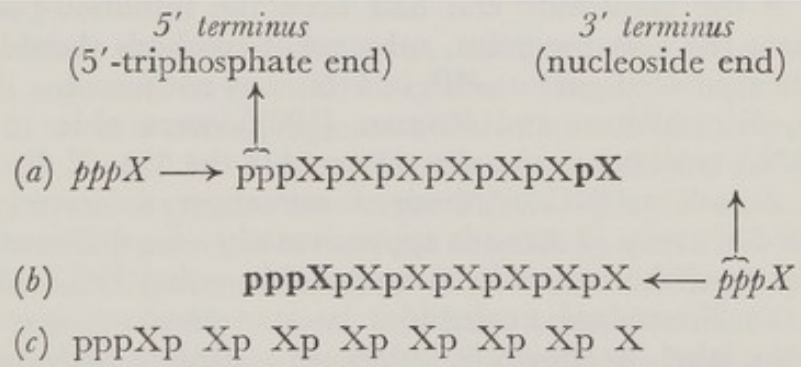


FIGURE 14.5 The diagram shows the two alternative ways in which RNA synthesis might occur. X stands for any of the 4 ribonucleosides and p before X means the 5′-phosphate and after X the 3′-phosphate (see § 12.5). The initial nucleotide is shown in bold type and the last in italics. (*a*) Growth in the 3′ to 5′ direction by addition of the ribonucleoside triphosphate (pppX) to the 5′ terminus, with release of pyrophosphate (pp) from the previous nucleoside triphosphate. (*b*) Growth in the 5′ to 3′ direction by addition of pppX to the 3′ terminus, with release of pyrophosphate from that triphosphate. (*c*) Components after alkaline hydrolysis, irrespective of direction of synthesis.

RNA was synthesized *in vitro* by Bremer, Konrad, Gaines and Stent (1965) using DNA-dependent RNA polymerase obtained from *Escherichia coli*, the four nucleoside 5′-triphosphates as substrate, and the DNA of virus *T*4 as primer. The adenosine 5′-triphosphate (ATP) had been labelled with tritium (^{3}H) in the adenine. During the RNA synthesis a 50-fold excess of unlabelled ATP was added. The nucleotides at each end of the synthetic RNA molecules were separated from one another and from the intervening nucleotides by alkaline hydrolysis to give the 2′- and 3′-phosphates, followed by paper electrophoresis. The separation of the nucleotides is possible because of their diversity of composition: from the 5′ end of each molecule there will be a nucleoside tetraphosphate (pppXp), from the 3′ end a nucleoside (X), and from the intervening region nucleoside monophosphates (Xp), where X stands for any of the four ribonucleosides and p before X means the 5′-phosphate and after X the 3′-phosphate—see Fig. 14.5(*c*). Bremer *et al.* found that the radioactivity of adenosine from the 3′ terminus was reduced after dilution of the labelled ATP, but the radioactivity of adenosine tetraphosphate from the 5′ terminus stayed constant. They inferred that in the growing molecule the 5′ terminus is constant, and additions are made to the 3′ terminus or, in other words, RNA synthesis proceeds from the 5′ to the 3′ end of the molecule (Fig. 14.5(*b*)).

Maitra and Hurwitz (1965) reached the same conclusion from a different experiment. Guanosine 5′-triphosphate (GTP) labelled with phosphorus-32 in its gamma phosphate (that farthest from the ribose) was incorporated into RNA *in vitro* for 5 min. using DNA from various sources in turn as primer, and then a 30-fold excess of unlabelled GTP was added. Continuing RNA synthesis did not diminish the amount of ^{32}P already incorporated, as expected if the initial nucleoside incorporated into RNA retained its 5′-triphosphate and growth was at the nucleoside or 3′ end (Fig. 14.5(*b*)). Conversely, if the nucleoside end had been the initiation-point and the 5′-triphosphate the growing-point, subsequent synthesis should release the previously incorporated gamma-^{32}P, but this was not found.

Goldstein, Kirschbaum and Roman (1965) were able to show that messenger RNA synthesis *in vivo* also occurred in the 5′ to 3′ direction. They found that *E. coli* at 0°C synthesized messenger sufficiently slowly—1 nucleotide added every 13 seconds approximately—for differential labelling of the two ends of the molecule to be possible, using ^{14}C-uridine. Radioactivity in the 3′-terminal nucleosides became constant very early after addition of the label, as shown by subsequent alkaline hydrolysis, whereas radioactivity in the internal nucleotides increased steadily, as expected if nucleotides are added to the 3′ end. Progressively more and more of the internal nucleotides would be labelled as synthesis proceeded after addition of the label, while the nucleoside ends would all become labelled at once, assuming the nucleotide pool was small.

Evidence is presented in § 14.15 that the direction of translation of the nucleotide sequence in RNA into the amino-acid sequence in a polypeptide is the same as the direction of transcription from DNA into RNA.

§ 14.9 The initiation of transcription

Bremer, Konrad, Gaines and Stent (1965) found, using RNA polymerase *in vitro* with the DNA of virus *T*4 of *Escherichia coli* as primer, that adenosine triphosphate was preferentially used for chain initiation compared with the other three nucleosides. Similarly, Maitra and Hurwitz (1965) found that adenine was the most frequent 5′-terminal base of the RNA when the DNA of *E. coli* viruses *T*2 and *T*5 was the primer, but that guanine could also occur in this position and was more frequent than adenine when DNA of *E. coli* or two other bacteria or of *Bos* was used as primer. This discovery that the 5′ end of the messenger frequently, and perhaps always, has a purine as base means that in the DNA chain which is transcribed a pyrimidine is preferentially chosen rather than a purine as the initiation-site for the synthesis of RNA. Maitra and Hurwitz suggested that a run of pyrimidines in DNA might be the signal for initiating transcription of that chain.

Support for this hypothesis was obtained by Kubinski, Opara-Kubinska and Szybalski (1966). They found that when the DNA of *Bacillus subtilis* was heated to separate the chains and then mixed with a synthetic RNA containing only guanine as base, one of the two chains of the DNA combined with the RNA and the other did not, allowing them to be separated in a caesium chloride gradient. It was inferred that the chain which formed a hybrid with RNA contains runs of cytosines. The significant observation was that this DNA chain, and not the other, also hybridized with messenger RNA from the bacterium, implying that the DNA chain with cytosine clusters was also the one which took part, predominantly at least, in transcription. Similar results were obtained with viruses *T*1 and *T*7 of *E. coli*. It is known that in virus $\phi X174$ (see § 14.7) and some other bacterial viruses the messenger RNA molecules are all transcribed from the same DNA chain.

In some other organisms, however, evidence has been obtained for cytosine-rich segments in both DNA chains. The hypothesis then predicts that there will be transcribing regions on both chains. This has been confirmed for virus λ of *E. coli* by Taylor, Hradecna and Szybalski (1967), who hybridized various λ-specific messenger RNAs with the chains of λ DNA which had been separated in a caesium chloride gradient, as they differ in density. Lambda DNA contains about 47,000 nucleotide-pairs, enough for some 40–50 genes, of which about half have been identified and mapped; moreover, Hogness, Doerfler, Egan and Black (1967) have established the orientation (that is, the direction of messenger synthesis) of several of these genes from genetic studies. Taylor *et al.* found, by the use of λ mutants deficient for particular regions of the chromoneme, that during the development of the virus a regular pattern of RNA synthesis occurred involving specific segments of each chain at specific times (Fig. 14.6). Their results were in agreement with those of Hogness *et al.* Moreover, the segments found to be transcribing regions were independently shown by their ability to hybridize with guanine-rich RNA to be segments containing cytosine clusters.

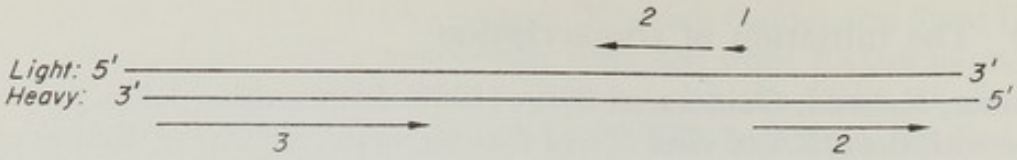


FIGURE 14.6 Diagrammatic representation of the light and heavy chains of the DNA of virus λ of *Escherichia coli* to show the transcription regions at different stages in the life-cycle of the virus:
(*1*) non-induced state, that is, the virus integrated into the host chromoneme; (*2*) early-acting genes; and (*3*) late-acting genes.
The arrows show the direction of messenger synthesis. The arrows labelled 2 and 3 each represent a number of different messenger RNA molecules.

It is evident that the hypothesis that pyrimidine clusters are the sites for the start of transcription, although not conclusively established, has considerable evidence to support it. The site of initiation of transcription has been called the *promoter*, and in one instance has been identified genetically (see § 15.6).

§ 14.10 The adaptor theory

In considering how base sequence in DNA or RNA could be translated into amino-acid sequence in a polypeptide chain, Crick (1957) pointed out that it was difficult to see how this was possible by direct contact between the coding nucleotides and the specific amino-acid. He predicted that each amino-acid was likely first to be combined with its own specific *adaptor*, and that each of these adaptors would have a highly specific spatial pattern of hydrogen bonds capable of associating it with a particular sequence of bases in DNA or RNA. He further suggested that molecules based on sequences of nucleotides were likely candidates for the adaptors, and that the combination between each amino-acid and its own specific adaptor could be made by a special enzyme. Within a year, Hoagland, Zamecnik, and Stephenson (1957) had discovered the existence of molecules of precisely the kind Crick had anticipated. They had found a low molecular weight RNA in the non-particulate fraction of an extract of the cytoplasm of liver cells of *Rattus norvegicus*. These RNA molecules, at first called soluble RNA to distinguish them from the RNA of the ribosomes, appeared to have the ability to bind amino-acids to themselves and to transfer them to peptide linkages. The names acceptor RNA and *transfer RNA* were subsequently proposed, because of this ability to accept amino-acids and transfer them to protein.

The primary steps in the process of protein synthesis, which were quickly established following the discovery of transfer RNA, appear to be as follows. Each amino-acid is first combined with adenosine triphosphate (ATP) and a specific enzyme to form a single complex consisting of the amino-acid, adenosine monophosphate (AMP) and the enzyme, with the elimination of pyrophosphate (Fig. 14.7(i)). Each of the 20 amino-acids has its own specific activating enzyme.

The second step is the interaction of the whole amino-acid–AMP–enzyme complex with a specific transfer RNA to form an amino-acid–RNA compound called an *amino-acyl transfer RNA*; AMP and the enzyme (amino-acyl transfer RNA synthetase) are released in the process (Fig. 14.7(ii)). There is at least one unique transfer RNA molecule for each amino-acid. It is customary to denote a transfer RNA by the name of its amino-acid

(i) $E_1 + aa_1 + Appp \longrightarrow E_1(aa_1 \cdot pA) + pp$

(ii) $E_1(aa_1 \cdot pA) + RNA_1 \longrightarrow aa_1 \cdot RNA_1 + E_1 + Ap$

(iii) $aa_1 \cdot RNA_1 + aa_2 \cdot RNA_2 \xrightarrow[Gppp]{Tr} aa_1 \cdot aa_2 \cdot RNA_2 + RNA_1$

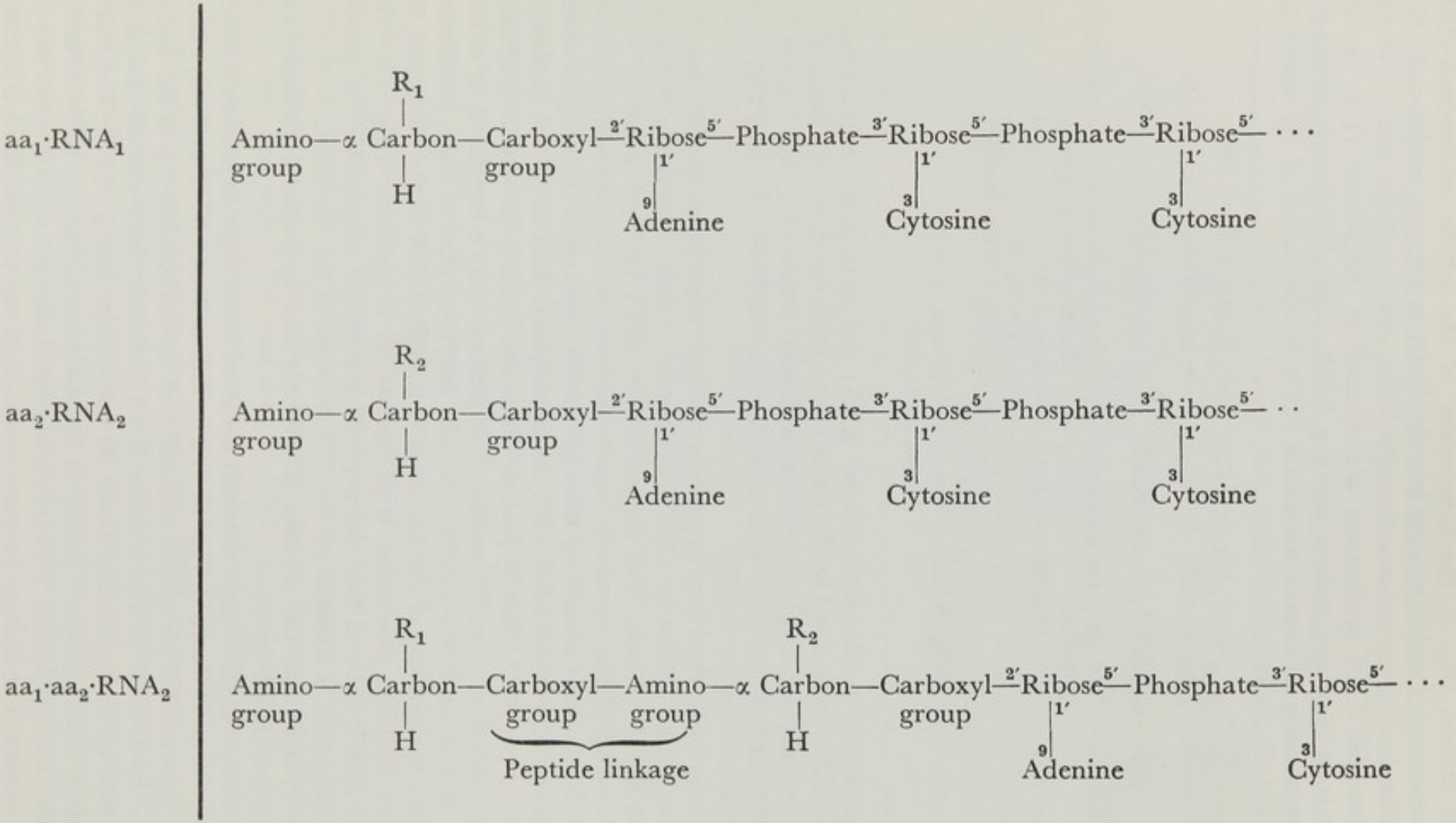
FIGURE 14.7 The three primary steps by which amino-acids are incorporated into proteins.
A = Adenosine (adenine ribonucleoside)
aa_1, aa_2 = Amino-acids nos. 1, 2
E_1 = Activating enzyme no. 1
G = Guanosine (guanine ribonucleoside)
p = Phosphate
RNA_1, RNA_2 = Transfer RNA nos. 1, 2
Tr = Transfer enzymes

as a noun (e.g. methionine transfer RNA) and to indicate the amino-acid–transfer RNA compound by the name of the amino-acid either as an adjective or abbreviated and hyphenated (e.g. methionyl transfer RNA or met-*t*RNA). Where more than one *t*RNA exists for the same amino-acid (see § 14.15) they are distinguished by a suffix.

The third and final step in protein synthesis takes place on the ribosomes. Takanami and Okamoto (1963) discovered, using a synthetic RNA containing uracil as base, that the messenger RNA becomes attached to the 30*S* sub-unit of the ribosome. Each transfer RNA–amino-acid complex evidently becomes associated through specific hydrogen bonding of certain of its nucleotides (*anticodon*) with the appropriate base sequence (codon) in the messenger RNA, thereby placing the amino-acids in the sequence dictated by the messenger. The formation of the peptide links is mediated by at least three (amino-acid non-specific) transfer enzymes. One of these transferases catalyzes the binding of the amino-acyl transfer RNA to the ribosome; this reaction requires guanosine triphosphate as a co-factor (Fig. 14.7(iii)). Monro (1967) showed that the peptidyl transferase responsible for peptide bond formation is situated on the 50*S* sub-unit of the ribosome.

It is evident that the transfer RNA molecules correspond precisely with Crick's postulated adaptors.

The nature of the chemical bonds involved in the steps in protein synthesis is shown in more detail in Fig. 14.8. The link between AMP and the amino-acid is between the phosphate of AMP and the carboxyl group of the amino-acid (—P—O—C—). The transfer RNA molecules each consist of a single nucleotide chain containing about 80 nucleotides (see § 14.11). The end of this chain which becomes linked to the amino-acid has the same structure in all the transfer RNAs. The terminal base is adenine and the next two are both cytosine. The linkage between amino-acid and transfer RNA is between


aa1·RNA1
Amino group
α Carbon
R1
H
Carboxyl group
2′ Ribose 5′
Phosphate
3′ Ribose 5′
Phosphate
3′ Ribose 5′
1′
9 Adenine
3 Cytosine
3 Cytosine
aa2·RNA2
R2
aa1·aa2·RNA2
Carboxyl group
Amino group
Peptide linkage

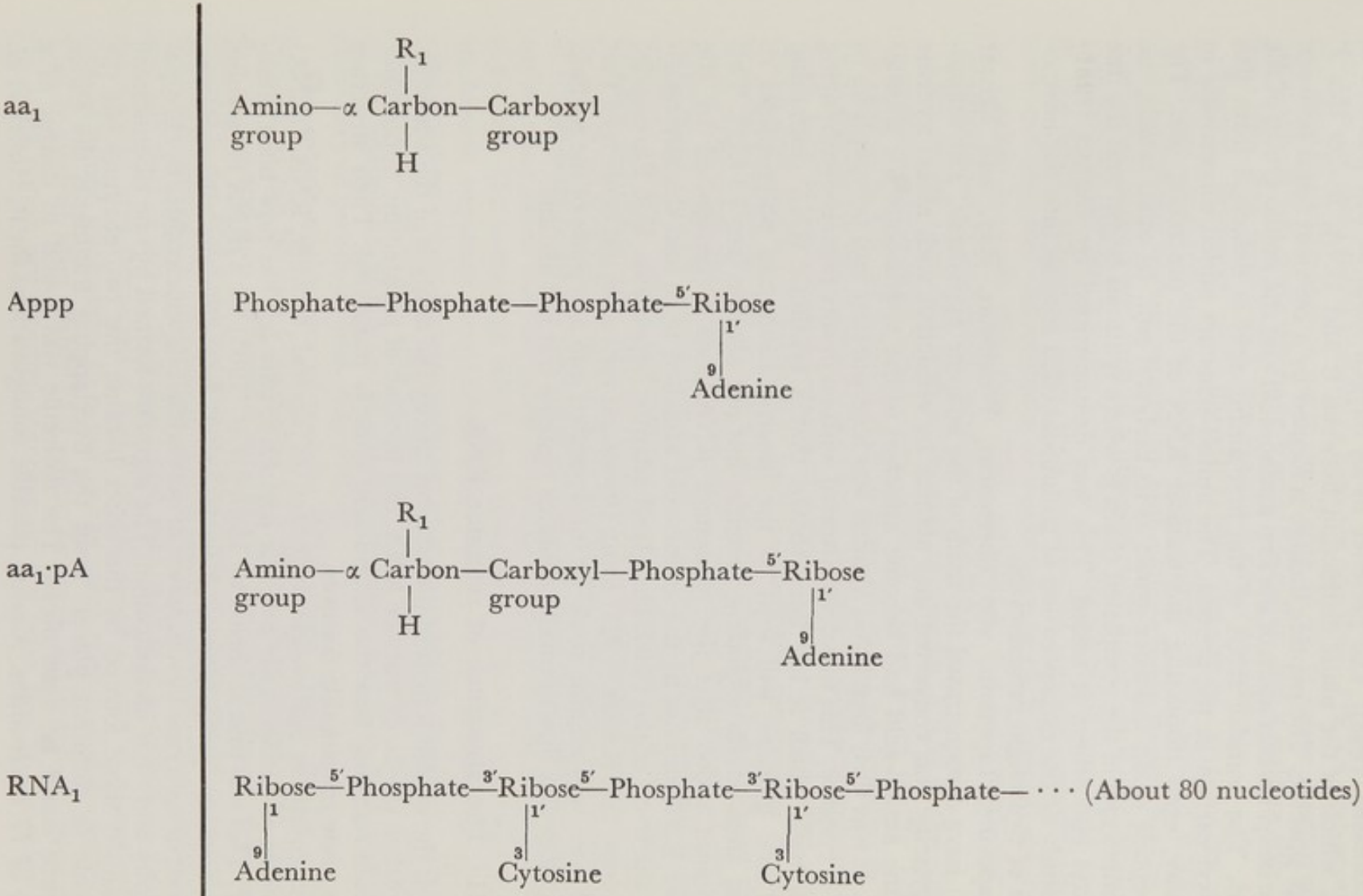


FIGURE 14.8 The structure of the intermediates in protein synthesis. The link between transfer RNA and amino-acid is shown as arising through the 2′ carbon atom of ribose but this is not yet conclusively established. The symbols in the left hand column have the same meaning as in Fig. 14.7.

the ribose of this terminal nucleotide (adenylic acid) and the carboxyl group of the amino-acid (—C—O—C—). It is probable, though not yet conclusively established, that the link arises through the 2′ carbon atom of the ribose (cf. Zamecnik 1962). The presence of an oxygen atom at this position in RNA, which is the main distinction between it and DNA, makes this 2′ linkage possible. The peptide linkage is formed by a covalent bond between the carboxyl carbon atom of the first amino-acid and the amino group of the second. The transfer RNA of the first amino-acid is detached from this carboxyl carbon in the process. The amino-acids are added successively in this way, each displacing the transfer RNA of the preceding one. The polypeptide chain therefore grows at its carboxyl end. In other words, the free amino end of the completed chain is where synthesis began and the free carboxyl end where it ended. This was demonstrated by Dintzis (1961) from study of the incorporation of tritium-labelled leucine into the haemoglobin of *Oryctolagus* reticulocytes.

Chapeville, Lipmann, von Ehrenstein, Weisblum, Ray, and Benzer (1962) have demonstrated the truth of the adaptor hypothesis. The amino-acid cysteine was converted into alanine by reduction with nickel hydride after the amino-acid had become attached to the cysteine-specific transfer RNA. It was then found that alanine was incorporated into polypeptides in place of cysteine. This was demonstrated with an *in vitro* protein-synthesizing system with which it has been shown that a synthetic RNA containing uracil and guanine will lead to the incorporation into polypeptides of certain amino-acids including cysteine, but not alanine (see § 14.12). This experiment shows that the messenger RNA code is recognized by the transfer RNA and not by the amino-acid attached to it, and thereby establishes the truth of the Crick-Hoagland adaptor hypothesis. Von Ehrenstein, Weisblum, and Benzer (1963) using *Oryctolagus* reticulocytes, confirmed that in the same way alanine could be incorporated into a peptide of the α-chain of haemoglobin that normally contains cysteine but not alanine.

§ 14.11 The structure of transfer RNA

With the establishment of the adaptor theory the analysis of the adaptors, or in other words, of transfer RNA, has become of paramount importance for understanding how the amino-acid code is translated. Their structure shows some remarkable features.

The complete nucleotide sequence of several transfer RNA (*t*RNA) molecules of *Saccharomyces cerevisiae* and *Escherichia coli* has now been established. This was first achieved by Holley *et al.* (1965) for a *t*RNA for alanine in *S. cerevisiae* using partial digestion with two different ribonucleases, followed in each case by separation of the various oligonucleotides by chromatography and their identification. The segments formed by one ribonuclease overlapped those formed by the other because the two enzymes cut the molecule in different places: from the overlapping fragments the entire sequence could be determined. The molecule was found to consist of a chain of 77 nucleotides. Among possible configurations for it, Holley *et al.*

suggested a clover-leaf shape (Fig. 14.9) because there were several places where a sequence of about 5 nucleotides was followed, after a region of unpaired nucleotides, by the complementary sequence in reverse order. Study of other *t*RNA molecules has given support for the clover-leaf model, but has revealed slight variations between one kind of *t*RNA and another in the lengths of the various parts (see Table 14.4). It seems, moreover, that although *t*RNAs normally have 4 arms (3 leaflets and a stalk to the clover-leaf) they may have a fifth (see Fig. 14.9 and Table 14.4).

Sanger, Brownlee and Barrell (1965) developed a two-dimensional technique for fractionating ribonuclease digests of ^{32}P-labelled RNA. The oligonucleotides were separated by high-voltage ionophoresis, first on strips of cellulose acetate and then in a plane at right angles on diethylaminoethyl (DEAE) paper, the transfer being by a blotting technique. The positions of the oligonucleotides were then determined autoradiographically, the spots cut out, and the sequence of nucleotides in each fraction ascertained by further enzymic digestion. This technique has facilitated the rapid determination of nucleotide sequences in RNA.

One of the most remarkable features of transfer RNAs is the diversity of unusual nucleotides which they contain. Those found in the 7 *t*RNAs referred to in Fig. 14.9 and Table 14.4 are listed (as nucleosides) in Table 14.5. Altogether these molecules contain at least 20 different minor nucleotides, such as methylated purines of various kinds, or nucleosides with the ribose methylated. One of the most remarkable nucleotides is pseudouridylic acid which has uracil as its nitrogenous base but with the linkage to the ribose at position 5 in the uracil molecule instead of at position 3 (see Fig. 14.10). The nucleoside so formed is called 5-ribosyluracil or pseudouridine. Lipsett (1965) discovered that certain *t*RNAs of *E. coli* contain sulphur as 4-thiouracil: two *E. coli* nucleosides containing sulphur are listed in Table 14.5, though not yet fully identified. The extraordinary diversity of nucleotides in *t*RNA simulates protein with its 20 different components rather than other nucleic acids. Nevertheless there is evidence that *t*RNA is formed like messenger RNA and ribosomal RNA by transcription of a nucleotide sequence from DNA (see § 18.7). The methylation or other modifications occur subsequently by means of specific enzymes: see Srinivasan and Borek (1966) and Goldwasser and Heinrikson (1966) for reviews of the enzymatic alteration of macromolecular structure, and of the biochemistry of pseudouridine, respectively.

The minor nucleotides of transfer RNA occur chiefly in the unpaired regions, as can be seen from Table 14.5 where these regions are lettered *b*, *d*, *f*, *h*, *j*, *n* and *p* to correspond with Fig. 14.9 and the paired segments are lettered *c*, *g*, *i* and *o*. Minor nucleotides have not been found near the place of attachment of the amino-acid, that is, the 3′-hydroxyl end of the molecule and the part of the 5′-phosphate end with which it is paired to form the amino-acid arm (segments *a* and *s* in Fig. 14.9). There is a tendency for certain minor nucleotides to occur in the same region of different *t*RNAs. This is evident from Table 14.5 where for each nucleotide occurrences in the same region are grouped together on the same line of the table. It is

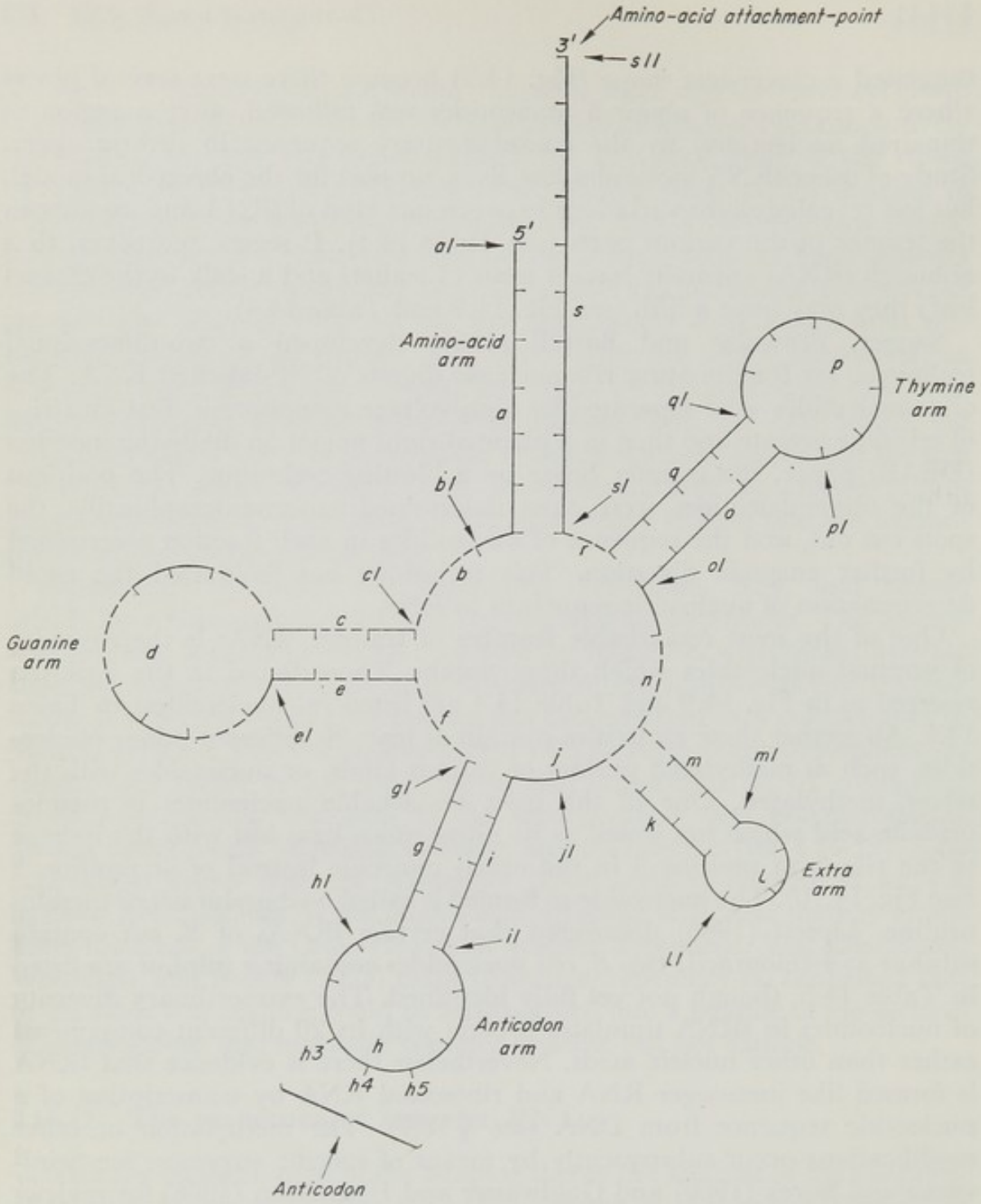


FIGURE 14.9 This is a diagrammatic representation of the structure of transfer RNA. The diagram is based on the nucleotide sequences of 7 *t*RNAs: for alanine, serine, tyrosine, valine and phenylalanine of *Saccharomyces cerevisiae*, and for tyrosine and formyl-methionine (see § 14.16) in *Escherichia coli*, from the results of Holley *et al.* (1965), Zachau, Dütting and Feldmann (1966), Madison, Everett and Kung (1966), Bayev *et al.* (1967), RajBhandary *et al.* (1967), Goodman *et al.* (1968) and Dube *et al.* (1968), respectively. The segments of the molecule are lettered in the 5′ to 3′ direction, that is, from the 5′-phosphate to the 3′-hydroxyl end of the chain, and the position of any nucleotide can be denoted by numbering them within each segment, also in the 5′ to 3′ direction: the first nucleotide of most of the segments is numbered in the diagram. Regions where the chain in one *t*RNA differs in length from that in another are indicated by broken lines. Regions of complementary pairing are shown by parallel lines.

TABLE 14.4 The table shows the numbers of nucleotides in the various segments of 7 different transfer RNAs from the results of the authors given in the caption to Fig. 14.9. The letters *a* to *s* refer to Fig. 14.9.

Segment of Molecule	*Saccharomyces cerevisiae* Transfer RNA for ala.	ser.	tyr.	val.	phe.	*Escherichia coli* Transfer RNA for tyr.	f.met.
Amino-acid arm							
a 5′ side	7	7	7	7	7	7	7
b Link to guanine arm	2	2	2	2	2	1	2
Guanine arm							
c Stem—5′ side	4	3	3	3	4	4	4
d Loop	10	10	12	11	8	11	9
e Stem—3′ side	4	3	3	3	4	4	4
f Link to anticodon arm	1	1	1	1	1	0	1
Anticodon arm							
g Stem—5′ side	5	5	5	5	5	5	5
h Loop	7	7	7	7	7	7	7
i Stem—3′ side	5	5	5	5	5	5	5
j Link* to *k* or *n*	2	1	3	1	3	1	3
Extra arm							
k Stem—5′ side	0	4	0	0	0	3	0
l Loop	0	3	0	0	0	3	0
m Stem—3′ side	0	4	0	0	0	3	0
n Link* to thymine arm	2	2	2	3	2	3	2
Thymine arm							
o Stem—5′ side	5	5	5	5	5	5	5
p Loop	7	7	7	7	7	7	7
q Stem—3′ side	5	5	5	5	5	5	5
r Link to amino-acid arm	0	0	0	1	0	0	0
Amino-acid arm							
s 3′ side	11	11	11	11	11	11	11
TOTAL	77	85	78	77	76	85	77

* When the extra arm (*k–m*) is absent *j* comprises purines and *n* pyrimidines.

evident also from the table that most of the minor nucleosides found in the *E. coli* *t*RNAs also occur at the same positions in at least one *t*RNA of *S. cerevisiae*, suggesting that these nucleotides may have persisted unchanged in position over the immense period of time since the chromonemal and chromosomal kingdoms diverged in evolution. It is clear, however, from the table that rather few of the minor nucleotides (indeed only two—and these neighbours) have been found at the same position in all 7 *t*RNAs. Nevertheless, when the normal nucleotides are also considered a fair number of constant features are evident (see Table 14.6). The -CCA sequence at the 3′-hydroxyl terminus was mentioned in § 14.10. Other constant features

TABLE 14.5 The table shows the minor nucleosides found in transfer RNAs for alanine, serine, tyrosine, valine and phenylalanine in *Saccharomyces cerevisiae* and for tyrosine and formylmethionine in *Escherichia coli* (for references see Fig. 14.9). The position of each nucleoside is indicated by the lettering and numbering system of Fig. 14.9, with identical or neighbouring positions for a nucleoside placed on the same line of the table.

Nucleoside	Position in Transfer RNA						
	Saccharomyces cerevisiae					*Escherichia coli*	
	ala.	ser.	tyr.	val.	phe.	tyr.	f.met.
1. *Purine nucleosides*							
Unidentified					*h*6		
(a) *Adenosine derivatives*							
1-Methyladenosine			*p*5	*p*5	*p*5		
N^6-Dimethyladenosine			*h*6				
N^6-Isopentenyladenosine		*h*6					
Unidentified (containing sulphur)						*h*6	
(b) *Guanosine derivatives*							
1-Methylguanosine	*b*2			*b*2			
N^2-Methylguanosine			*c*1		*c*1		
N^2-Dimethylguanosine	*f*1	*f*1	*f*1		*f*1		
N^7-Methylguanosine					*j*3		*j*3*
2′-O-Methylguanosine		*d*5	*d*6			*d*5	
					*h*3		
Unidentified						*h*3	
Inosine	*h*3	*h*3		*h*3			
1-Methylinosine	*h*6						
2. *Pyrimidine nucleosides*							
(a) *Cytidine derivatives*							
N^6-Acetylcytidine		*c*3					
5-Methylcytidine					*i*2		
		*n*2	*n*2	*n*3	*o*1		
2′-O-Methylcytidine					*h*1		*h*1
(b) *Uridine derivatives*							
Ribothymidine	*p*1	*p*1	*p*1	*p*1	*p*1	*p*1	*p*1
5,6-Dihydrouridine	*d*5	*d*4	*d*4, 5	*d*4	*d*3, 4		
	*d*9	*d*7, 8	*d*8, 9, 10	*d*8, 9			*d*8
	*n*1*		*n*1	*n*2			
4-Thiouridine						*b*1†	*b*1
2′-O-Methyluridine		*j*1					
Pseudouridine				*d*1			
				*g*1			
		*h*1		*h*1			
			*h*4				
	*h*7	*i*1	*i*1		*i*1	*i*1	
	*p*2	*p*2	*p*2	*p*2	*p*2	*p*2	*p*2

* This nucleoside occurs in one form of the *t*RNA but not in another: see § 14.14.

† Identification of nucleoside tentative.

Takemura *et al.* (1968) found that a valine *t*RNA from the yeast *Torulopsis utilis* differed from valine *t*RNA I of *S. cerevisiae* at *d*5 (5,6-dihydrouridine), *d*9 (cytidine), *n*2 (5-methylcytidine), *n*3 and *r*1 (no nucleotides: total 75). Staehelin *et al.* (1968) found that a serine *t*RNA from *Rattus norvegicus* differed from serine *t*RNA II of *S. cerevisiae* in 20 nucleosides: *h*1 and *l*2 (both 3-methylcytidine), *i*1 (2′-O-methylpseudouridine), *p*5 (1-methyladenosine) and the complementary pairs at *a*2–5, *a*7, *g*2 (pseudouridine), *g*3 and *k*4.

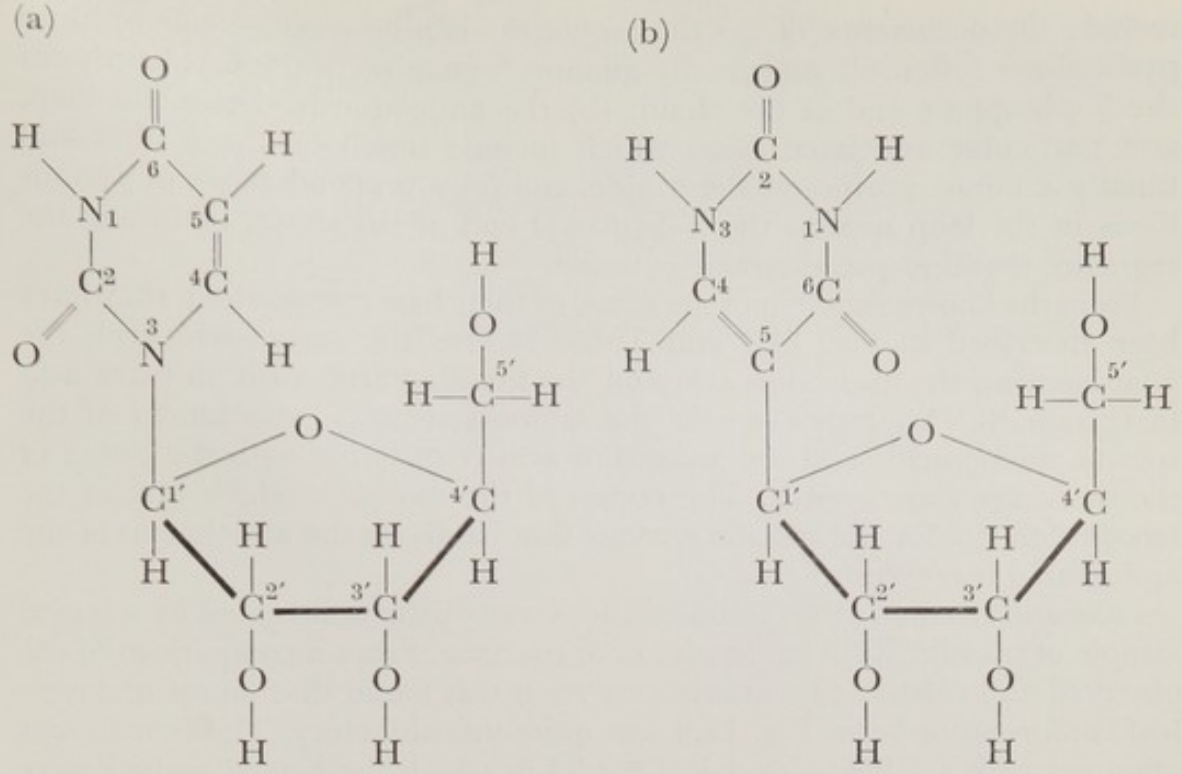


FIGURE 14.10 The structure of (a) uracil ribonucleoside (uridine), and (b) 5-ribosyluracil (pseudouridine).

TABLE 14.6 The table shows the nucleosides which occur constantly at certain positions in 7 transfer RNAs (for alanine, serine, tyrosine, valine and phenylalanine in *Saccharomyces cerevisiae* and for tyrosine and formylmethionine in *Escherichia coli*; for references see Fig. 14.9). The position of the nucleosides is indicated by the lettering and numbering system of Fig. 14.9.

Position	*Nucleoside*
*b*1	Uridine*
*b*2†	With a purine as base*
3′ end of *c*	Cytidine
Sequence in *d* starting at *d*1, *d*2 or *d*3	Adenosine
	Guanosine
	1, 2 or 3 nucleosides all with a pyrimidine as base, often 5,6-dihydrouridine
	Guanosine*
	Guanosine
*e*1	Guanosine
*h*1	With a pyrimidine as base
*h*2	Uridine
(*h*3–5	Anticodon)
*h*6	With a minor purine as base‡

Position	*Nucleoside*
3′ end of *n*	Cytidine*
*o*5	Guanosine
*p*1	Ribothymidine
*p*2	Pseudouridine
*p*3	Cytidine
*p*4	With a purine as base
*p*5	Adenosine*
*p*7	With a pyrimidine as base
*q*1	Cytidine
*s*8	With a purine as base
*s*9	Cytidine
*s*10	Cytidine
*s*11	Adenosine

* A derivative in one or more of the *t*RNAs (see Table 14.5).
† Not present in *E. coli* tyr. *t*RNA (see Table 14.4).
‡ Except in *S. cerevisiae* val. and *E. coli* f.met. *t*RNAs (see § 14.16).

include the occurrence of (a) the sequence adenine–guanine–one or more pyrimidines (often dihydrouracil)–guanine*–guanine in the first loop from the 5′-phosphate end of the chain, (b) the anticodon in the second loop, and particular associated bases which include uracil on the 5′ side and usually a minor purine on the 3′ side, and (c) a preponderance of pyrimidines in the loop nearest the 3′-hydroxyl end of the chain, including the sequence thymine–pseudouracil–cytosine.

From the known function of the arms, or their base composition, they have been described in Fig. 14.9 and Table 14.4 as the amino-acid arm, the guanine arm, the anticodon arm and the thymine arm, with an extra arm in certain *t*RNAs. Functions for the amino-acid arm (attachment of the specific amino-acid) and the anticodon arm (registering with the codon of the messenger) are known. The region of the molecule which acts as the recognition site for the specific enzyme that catalyzes the attachment of the amino-acid is not known.

Lake and Beeman (1968) studied the X-ray diffraction caused by a mixed sample of transfer RNA molecules of *S. cerevisiae*. From a comparison of the observed and calculated scattering curves it was found that an open clover-leaf conformation as in Fig. 14.9 was quite unsatisfactory. The best fit was obtained with a folded clover-leaf model in which the helical regions were fitted together as closely as possible and the arms lay in two pairs, the amino-acid and anticodon arms pointing in one direction and the guanine and thymine arms in the opposite direction with their loops close together as in Fig. 14.11. This configuration, of the many possible, was tested because Madison, Everett and Kung (1966) had suggested there might be base pairing between the guanine and thymine loops because of their resistance to ribonuclease. Another possibility, which Lake and Beeman found fitted the X-ray scatter slightly less well, was with 3 arms in one direction. They pointed out that the X-ray scattering data provided no evidence either for or against the clover-leaf model, but did indicate that *t*RNA is very compact such that, if of clover-leaf form, the leaflets are folded together.

Gilbert (1963) suggested there was only one active site for the synthesis of the peptide bond on each ribosome, and that each *t*RNA molecule takes the place of the preceding one, adding its amino-acid to the chain and ejecting the previous *t*RNA as it does so. In Fig. 14.11, an amino-acyl-*t*RNA is supposed just to have added amino-acid no. 12 to a polypeptide chain, thereby becoming a *peptidyl-tRNA* and displacing the *t*RNA which brought amino-acid no. 11. Gilbert suggested that this displacement of the previous tenant forces the messenger to move over the ribosome by one reading unit, thereby allowing the next amino-acyl-*t*RNA to take up position: in the diagram, this is the *t*RNA carrying amino-acid no. 13. Warner and Rich (1964), from study of haemoglobin synthesis in the reticulocytes of *Oryctolagus*, showed that each ribosome active in protein synthesis contained two *t*RNA molecules. They therefore modified Gilbert's proposals and suggested there were two adjacent active sites on the ribosome: one (called the *peptide site* by Bretscher and Marcker, 1966) for the peptidyl-*t*RNA carrying the growing

* Nucleoside methylated in the ribose in several *t*RNAs.

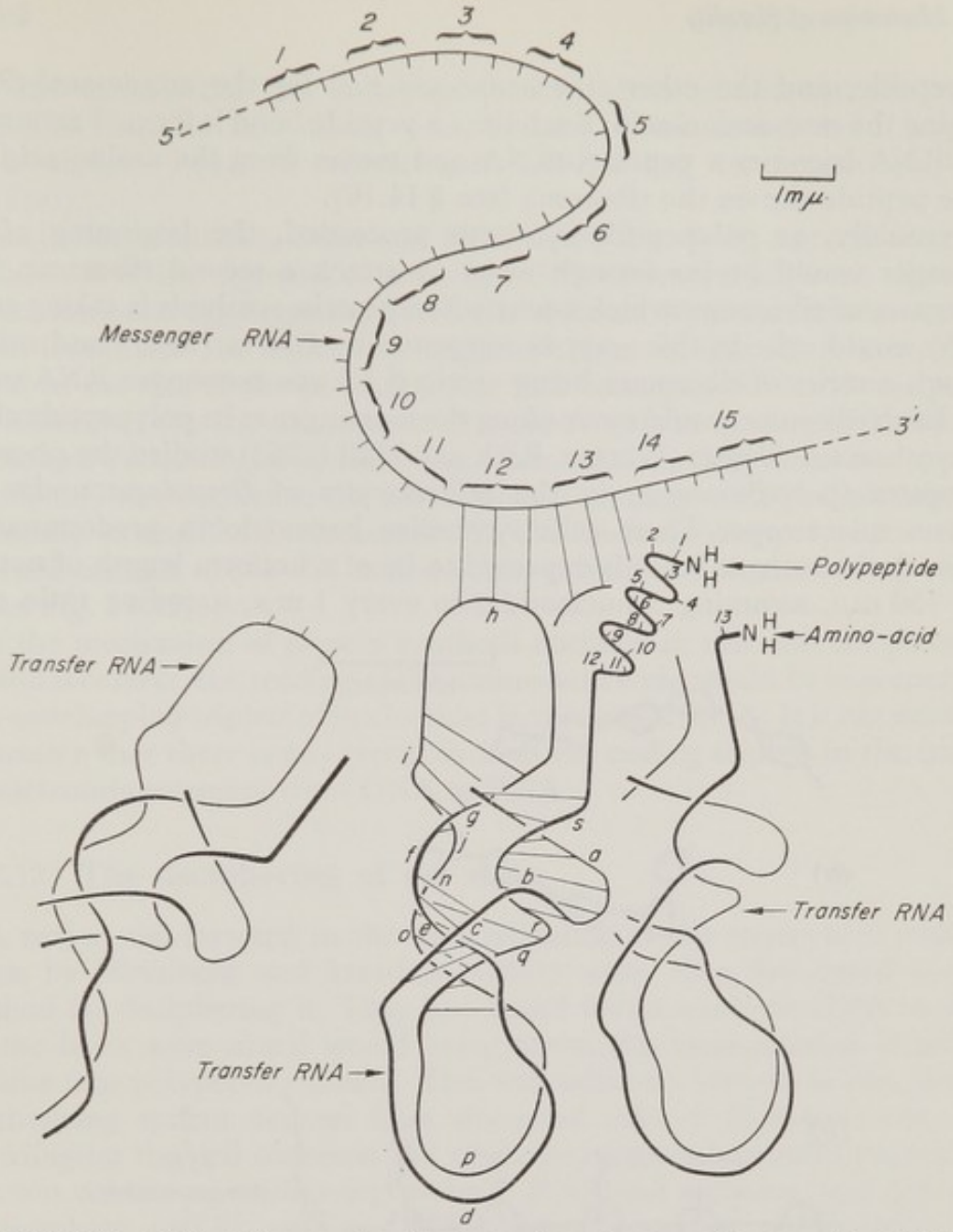


FIGURE 14.11 Diagram to illustrate the structure for transfer RNA proposed by Lake and Beeman (1968), and to show how the triplet sequence in messenger RNA determines which amino-acyl transfer RNA molecules shall succeed one another in taking up position on the ribosome (not shown), and hence which amino-acids shall succeed one another in the polypeptide. The transfer RNA which added amino-acid no. 11 to the polypeptide has just been displaced by that which has brought no. 12, while that bringing no. 13 has just been selected through the ability of its anticodon to pair with triplet no. 13 in the messenger. Lake and Beeman's model for transfer RNA is a folded clover-leaf (compare Fig. 14.9): the amino-acid arm is shown above and to the right of the anticodon arm and, at the other end, the larger guanine loop lies above the thymine loop. In one of the *t*RNAs the hydrogen bonding is shown, and the segments are lettered to correspond with Fig. 14.9.

polypeptide, and the other (the *amino-acid site*) for the amino-acyl-*t*RNA bringing the next amino-acid. Each time a peptide bond is formed an amino-acyl-*t*RNA becomes a peptidyl-*t*RNA and moves from the amino-acid site to the peptide site on the ribosome (see § 14.16).

Eventually, as polypeptide synthesis proceeded, the beginning of the messenger would be far enough away to attach a second ribosome. The aggregates of ribosomes which occur when protein synthesis is taking place (Pl. 5) would arise in this way, as suggested by Gierer (1963) and others, through a series of ribosomes being serviced by one messenger RNA molecule. Each ribosome would move along the messenger as its polypeptide chain was synthesized. Slayter, Warner, Rich and Hall (1963) studied the ribosome aggregates (polyribosomes) in the reticulocytes of *Oryctolagus* under the electron microscope. These cells synthesize haemoglobin predominantly, and so the messenger RNA is expected to be of a uniform length of not less than 150 mμ, assuming 3 nucleotides to every 1 mμ, a coding ratio of 3,

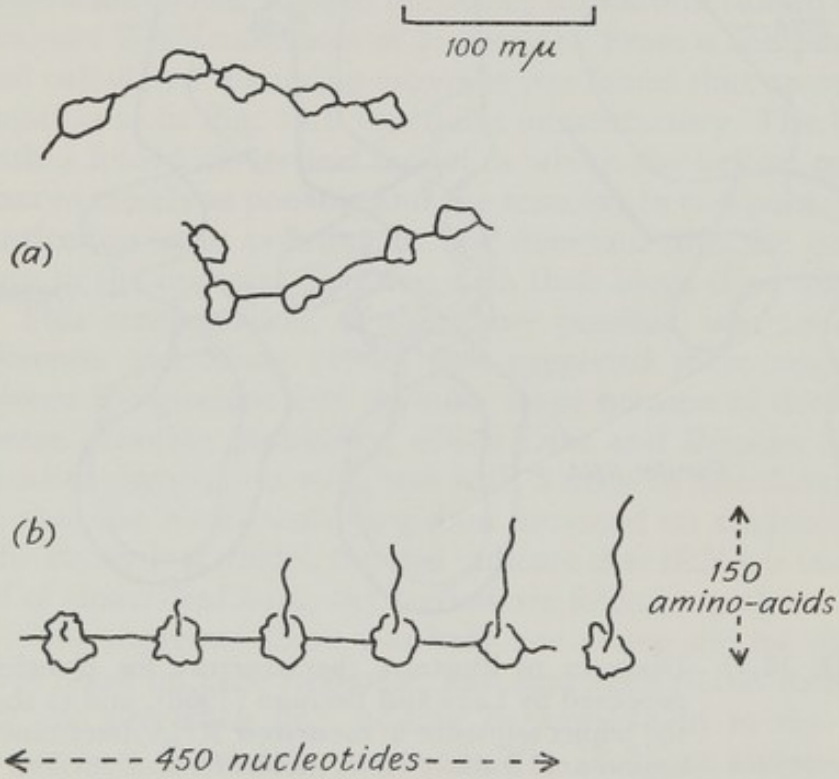


FIGURE 14.12 Ribosomes from reticulocytes of *Oryctolagus cuniculus* (Rabbit). The thread joining the ribosomes is thought to be messenger RNA for one of the polypeptide chains of haemoglobin.

(*a*) Appearance as seen in electron micrographs prepared by Slayter *et al.* (1963).

(*b*) Diagrammatic representation to show the progressive formation of the polypeptide chain on the ribosomes as they move along the messenger RNA (from left to right in the diagram).

and 150 amino-acids per polypeptide. The electron micrographs agreed with this expectation; there were usually 5 ribosomes held together by a thread 1 mμ in width and 170–280 mμ in length (see Fig. 14.12(*a*)).

Staehelin, Wettstein, Oura and Noll (1964) found the average length in nucleotides of the segment of messenger RNA between adjacent ribosomes in the ribosomal aggregates of liver cells of *Rattus*. By sedimentation analysis it was found that the molecular weight of the messenger RNA was proportional to the size of the polyribosome, and corresponded to 90 nucleotides per ribosome. On the assumption that the ribosome spacing is the same in *Oryctolagus* reticulocytes synthesizing haemoglobin, there must be approximately 450 nucleotides to the haemoglobin messenger RNA molecule since it usually extends to 5 ribosomes. As the haemoglobin polypeptides synthesized contain about 150 amino-acids, it follows that the coding ratio is 3 (see Fig. 14.12(*b*)).

If the mechanism of protein synthesis outlined in this and the preceding section is correct, the reading of the amino-acid code would be in consecutive non-overlapping triplets of nucleotides in messenger RNA. It is not necessary to assume that there is any recognition of the coding triplets in the transfer of nucleotide sequence from DNA to RNA.

§ 14.12 The deciphering of the code

A major step forward in the understanding of the amino-acid code was taken by Nirenberg and Matthaei (1961) when they discovered a direct method for deciphering it. They had found that a synthetic RNA in which all the bases were uracil would bring about the incorporation of phenylalanine into polypeptide chains. This was achieved with an *in vitro* protein-synthesizing system derived from disrupted cells of *Escherichia coli*. After centrifuging the cell contents, the ribosomes were mixed with supernatant solution containing amino-acyl transfer RNA and enzymes, and adenosine triphosphate (ATP) and an ATP-generating system were added. The synthetic RNA was obtained by means of the polynucleotide phosphorylase first isolated by Grunberg-Manago and Ochoa (1955) (see § 14.4). The amino-acid was labelled with ^{14}C and the subsequent presence of the label in polypeptide chains demonstrated.

Lengyel, Speyer, and Ochoa (1961) synthesized an RNA using a mixture of the ribonucleoside diphosphates of uracil (U) and cytosine (C) in the proportion of 5:1. On the assumption that nucleotides are randomly incorporated into the chain in proportion to their abundance, out of every 216 triplets there will be 125 with the sequence UUU, 25 each with UUC, UCU, and CUU, 5 each with UCC, CUC, and CCU and 1 with CCC Lengyel *et al.* found that when added to the protein-synthesizing system, this RNA led to the incorporation of serine as well as phenylalanine into the polypeptide. There were approximately 4·4 phenylalanine residues to every 1 of serine, and so it was tentatively concluded that, if it is a triplet code, one of the 3 trinucleotides containing 2 uracils and 1 cytosine codes for serine. In the

same way, a synthetic RNA containing uracil and adenine in the proportion of 5:1 was found to lead to the incorporation of phenylalanine and tyrosine in the proportion of 4:1, suggesting that the code for tyrosine contained 2 uracils and 1 adenine.

By an extension of this technique, provisional nucleotide triplets (without knowledge of the base sequence) were soon assigned to nearly all the amino-acids. A notable feature was that all these triplets contained uracil. This suggested that more than one triplet could code for the same amino-acid, since uracil was not known to occur in such excess in natural messenger RNA. The multiple coding* for individual amino-acids was confirmed when Gardner, Wahba, Basilio, Miller, Lengyel, and Speyer (1962) found that most synthetic RNA molecules without uracil will also bring about amino-acid incorporation. Moreover, they showed that an RNA containing only adenine leads to a polypeptide composed of lysine residues. This was first demonstrated with an *in vitro* protein synthesizing system derived from liver cells of *Rattus*, and it was subsequently shown with the *E. coli* system. In conjunction with the original discovery that uracil RNA gives a phenyl-alanine polypeptide, this finding was of particular significance because adenine and uracil are complementary. The implication was that nucleotide sequence is transcribed from one particular chain of the DNA molecule, because the other chain would lead (through a complementary messenger RNA) to the formation of a different polypeptide. Thus an adenine triplet in DNA would give a uracil triplet in messenger RNA and hence phenyl-alanine in the peptide, while the complementary thymine triplet in the other DNA chain would give rise to an adenine triplet in messenger RNA and hence lysine in the peptide. As indicated in § 14.7 and § 14.9, there is experimental evidence that, in any particular region of the DNA, messenger RNA is indeed synthesized only as the complement of one specific chain of the DNA.

In confirmation of the alternative coding for individual amino-acids, Weisblum, Benzer and Holley (1962) demonstrated the existence in extracts of *E. coli* of two different transfer RNA molecules each specific for the same amino-acid (leucine). The two leucine transfer RNAs were separated by countercurrent fractionation. This technique depends on the discovery that certain *t*RNAs have widely different partition coefficients in a two-phase solvent system composed of pH 6 phosphate buffer, formamide and iso-propanol. It was found that incorporation of leucine into polypeptides by one of these *t*RNAs was stimulated by a synthetic RNA containing uracil and cytosine, and incorporation by the other was stimulated by a uracil–guanine RNA. Von Ehrenstein and Dais (1963) isolated a third leuinec transfer RNA which responded to an RNA containing uracil only.

Nirenberg and Leder (1964) described a method using *E. coli* extracts for measuring the interaction of transfer RNAs carrying their specific amino-acids with ribosomes carrying messenger RNA. The behaviour of

* Multiple coding for individual amino-acids is often referred to as code *degeneracy*, but, as Tatum (1964) has pointed out, this is a misleading expression, since it has evolutionary implications which may not be valid.

synthetic polyribonucleotides of various lengths and compositions, acting as messenger, was studied. Two enzymatic methods had been devised for synthesizing these oligonucleotides. Trinucleotides with a 5′-terminal phosphate were found to be highly active in binding specific amino-acyl transfer RNAs to ribosomes, whereas similar dinucleotides, and also trinucleotides with 3′-terminal phosphate, were largely or entirely inactive. The trinucleotides with 5′-terminal phosphate containing only uracil (UUU), only adenine (AAA) and only cytosine (CCC) were found to direct the binding to ribosomes of *t*RNAs for phenylalanine, lysine and proline, respec-

TABLE 14.7 The table shows the amino-acid code established from *in vitro* studies with *Escherichia coli*. The orientation of the trinucleotides follows the conventional sequence (cf. § 12.5), that is, with 5′-phosphates to the left and 3′-phosphates to the right of the symbols (A, C, G and U) for the ribonucleosides of adenine, cytosine, guanine and uracil.

UUU, UUC	Phenylalanine	UCU, UCC, UCA, UCG	Serine	UAU, UAC	Tyrosine	UGU, UGC	Cysteine
UUA, UUG	Leucine	CCU, CCC, CCA, CCG	Proline	UAA, UAG	Stop†	UGA	Stop†
CUU, CUC, CUA, CUG	Leucine	ACU, ACC, ACA, ACG	Threonine	CAU, CAC	Histidine	UGG	Tryptophan
AUU, AUC, AUA	Isoleucine	GCU, GCC, GCA, GCG	Alanine	CAA, CAG	Glutamine	CGU, CGC, CGA, CGG	Arginine
AUG	Methionine*			AAU, AAC	Asparagine	AGU, AGC	Serine
GUU, GUC, GUA, GUG	Valine			AAA, AAG	Lysine	AGA, AGG	Arginine
				GAU, GAC	Aspartic acid	GGU, GGC, GGA, GGG	Glycine
				GAA, GAG	Glutamic acid		

* Also, polypeptide initiation (see § 14.16).
† Polypeptide termination (see § 14.17).

tively, in agreement with earlier work. Studies with this trinucleotide binding technique quickly established the identity of a majority of the coding triplets and, coupled with the work of Khorana *et al.* (1967) using polynucleotides with known repeating di- and trinucleotide sequences, solved the code (see Table 14.7 from Morgan, Wells and Khorana, 1966).

The technique developed by Khorana and associates was to prepare short deoxyribopolynucleotides of known sequence by chemical methods, to use these synthetic polynucleotides as templates for DNA polymerase action to obtain high molecular weight DNA with known repeating nucleotide sequence, and then to use DNA-dependent RNA polymerase to obtain a synthetic messenger RNA also of known repeating sequence. It was found that for DNA polymerase to act it was necessary to have complementary sequences each of 8–12 nucleotides. The synthetic DNA formed had the duplex structure. It was prepared with not more than 3 different bases in the individual chains, so that by supplying only the appropriate ribonucleoside triphosphates the action of the RNA polymerase could be restricted to

one particular DNA chain. Thus, a synthetic DNA with the base sequence TAC repeated many times in one chain and with GTA repeated in the other gave rise to RNA with the repeating sequence GUA when the triphosphates of these 3 nucleosides were supplied, and with the sequence UAC in the presence of the triphosphates of U, A and C. Having obtained RNA with such known sequences of nucleotides, Nirenberg and Matthaei's technique of cell-free polypeptide synthesis was used to assign codons to amino-acids. In this way Nishimura, Jones, Ohtsuka, Hayatsu, Jacob and Khorana (1965) found that a ribopolynucleotide containing the repeating sequence AAG directed the synthesis of 3 different polypeptides containing lysine, arginine and glutamic acid, respectively. Which of the 3 was formed in any particular instance evidently depended on whether the reading-frame was set to read the message as AAG, AGA or GAA. Nishimura, Jones and Khorana (1965) found that RNA containing the nucleosides U and C in alternating sequence directed the synthesis of a polypeptide containing serine and leucine in alternating sequence. This is again what is expected from a non-overlapping triplet code read consecutively, because the messenger will have UCU alternating with CUC.

Khorana and associates also applied Nirenberg and Leder's technique of trinucleotide-stimulated binding of amino-acyl transfer RNAs to ribosomes, using ribotrinucleotides prepared by chemical methods. By this combination of techniques they not only established the total structure of the amino-acid code but obtained direct proof that the coding ratio is three, that the code is non-overlapping and that contiguous triplets are read sequentially without omission of any bases.

These experiments established the code for *Escherichia coli in vitro*. How far the same code applies *in vivo* and to other organisms is discussed in the next section.

§ 14.13 Mutations and the code

Study of the changes in polypeptides caused by mutations has provided information about the operation of the amino-acid code in living cells, such as has not been obtainable in any other way. Data are available for proteins in several diverse organisms, and all are in agreement with the code deduced from the cell-free studies described in § 14.12.

Tsugita and Fraenkel-Conrat (1962) and Wittmann (1962) studied the effects of nitrous acid in causing mutations affecting the coat protein of tobacco mosaic virus. Since nitrous acid is thought to cause mutation by altering cytosine to uracil, and adenine to hypoxanthine which is then expected to pair like guanine (see § 14.2), any changes in the protein of the virus coat caused by mutations induced with nitrous acid will be expected to be the result of one or other of these two base substitutions. These authors have found that particular amino-acid substitutions such as phenylalanine in place of serine, and isoleucine in place of threonine, turn up repeatedly with nitrous acid treatment. Later results are reviewed by Wittman and Wittman-Liebold (1967). As pointed out by Crick (1967), there is good agreement

between these substitutions and those predicted by changing cytosine to uracil or adenine to guanine in the code deduced from the results of protein synthesis *in vitro* using *Escherichia coli* components and synthetic RNA.

Yanofsky, Ito and Horn (1967) studied the changes due to mutations affecting the *A* protein of the enzyme tryptophan synthetase of *Escherichia coli* (see § 13.8 and Fig. 13.8). No less than 10 different amino-acid substitutions have been found at position 210 (Fig. 14.13(*a*)) and 4 at position 233 (Fig. 14.13(*b*)). Henning and Yanofsky (1962) and Guest and Yanofsky (1965) found that some of these strains yielded wild-type recombinants when crossed with one another, evidently through recombination within the nucleotides of DNA which provide the template for one codon in messenger RNA. The significant feature of the changes observed, either by mutation or recombination, is that they are all explicable in terms of single nucleotide changes, or single exchanges between nucleotides, on the basis of the *in vitro* code. The data thus support the code and indicate that mutations normally affect only one nucleotide.

Terzaghi, Okada, Streisinger, Emrich, Inouye and Tsugita (1966) have found that the enzyme lysozyme produced by the normal (wild-type) form

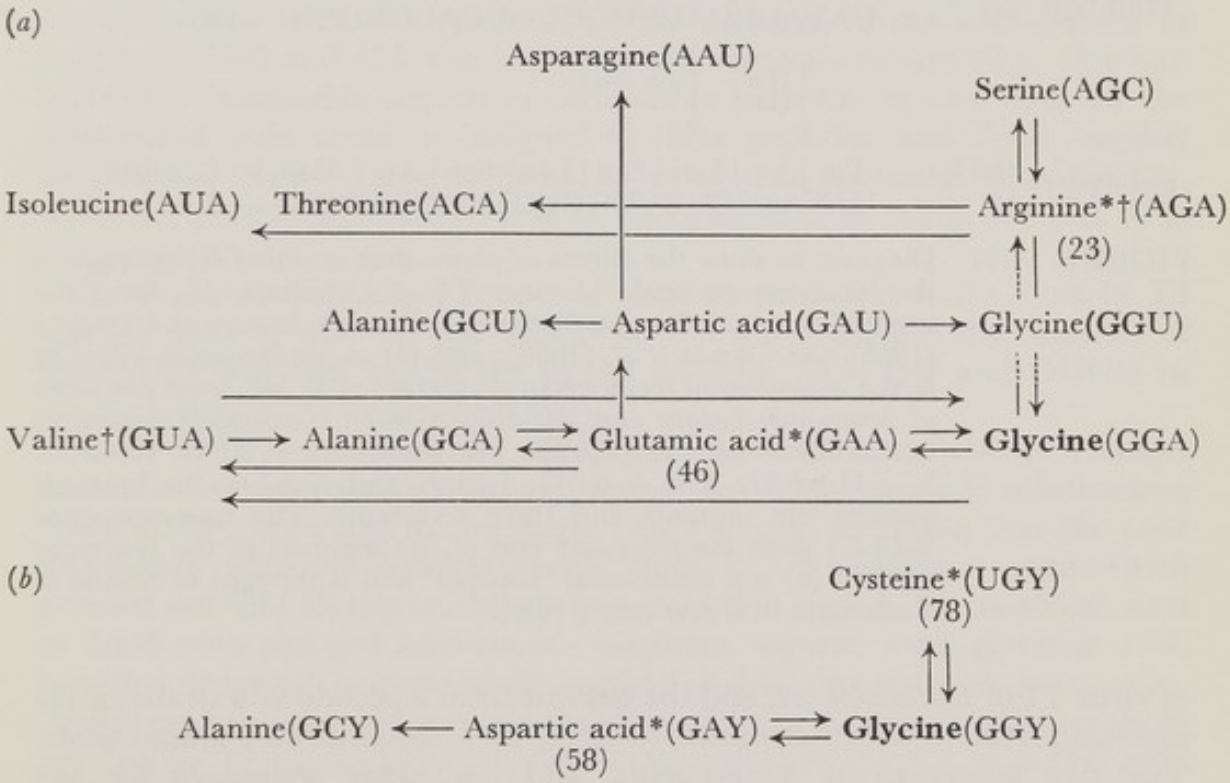


FIGURE 14.13 Diagram to show changes observed by Yanofsky and associates in the A protein of tryptophan synthetase of *Escherichia coli* as a result of mutation and recombination. The arrows show amino-acid changes observed through mutation (*a*) at position 210 and (*b*) at position 233 from the wild-type shown in heavy type. Numbered mutants correspond to Fig. 13.8; un-numbered mutants are full or partial revertants of these. Pairs of strains which have given wild-type recombinants on crossing are marked with asterisks or daggers. The probable RNA codons, on the basis of the *in vitro* code, are shown in brackets; Y = U or C.

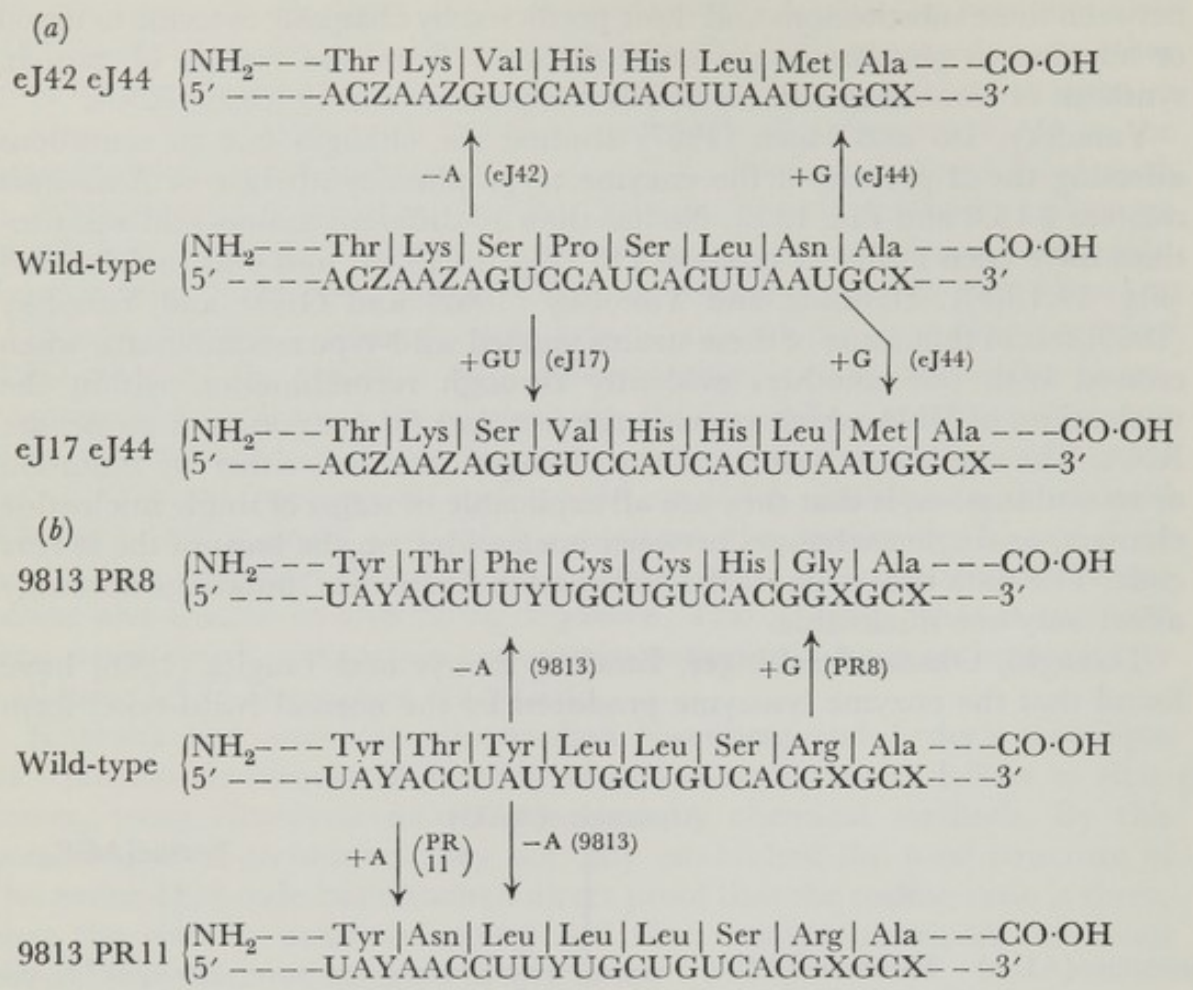


FIGURE 14.14 Diagram to show the effects of phase-shift mutants (*a*) on part of the lysozyme molecule of virus *T*4 of *Escherichia coli*, form the work of Terzaghi, Okada, Streisinger, Emrich, Inouye and Tsugita (1966) and Okada *et al.* (1966), and (*b*) on amino-acids 172–179 in the *A* protein of tryptophan synthetase of *E. coli*, from the work of Brammar, Berger and Yanofsky (1967). Nucleotide sequences compatible with the peptides are shown. X = A, C, G or U. Y = C or U. Z = A or G. Letters and numbers in brackets identify the mutants and their revertants. The base sequence *ZAAZA* near the left-hand end of the segment of the lysozyme gene in (*a*) is a mutational 'hot-spot' and is thought to consist of 5 adenines in a row (see § 16.6).

of virus *T*4 of *Escherichia coli* and the enzyme from a pseudowild strain carrying two proflavin-induced mutations differ by a sequence of 5 amino-acids. The two sequences of amino-acids, and nucleotide sequences for the messenger compatible with both of them, are shown in the upper part of Fig. 14.14(*a*). One proflavin mutant (eJ42) had evidently lost a particular adenine nucleotide and the other mutant (eJ44) had gained a guanine nucleotide 15 nucleotides further along the molecule. The double mutant had a nearly normal phenotype (pseudowild-type) because the reading-frame was evidently set in register again after only 5 triplets had been misread. The results not only suggest that the code established for *E. coli in vitro* applies to *T*4 *in vivo*, but they confirm the hypothesis of Crick and associates

(1961) that the translation of the genetic message is initiated at a given point where a reading-frame is set in register (see § 14.3). The results also confirm that proflavin acts as a mutagen by inserting or deleting nucleotides, that is, it causes phase-shift mutations—a shift in the reading-frame causing a grossly different translation of the message beyond the site of the mutation. A further aspect of this remarkable experiment is discussed in § 14.14.

Okada, Terzaghi, Streisinger, Emrich, Inouye and Tsugita (1966) made similar studies with another proflavin mutant (eJ17) which, like eJ42, was found to give a pseudowild phenotype when brought into the same molecule as eJ44 by recombination. The amino-acid sequence of this double mutant is shown in the lower part of Fig. 14.14(*a*). It differs from the other double mutant by the addition of one amino-acid (serine). Mutant eJ17 evidently arose from the addition of two nucleotides (GU or UG). In the double mutant the addition of G by mutant eJ44 then restores the normal setting of the reading-frame, but adds one extra amino-acid to the molecule. Other examples of phase-shift mutants in the lysozyme gene (see Fig. 18.3) of *T*4 are discussed by Streisinger, Okada *et al.* (1967).

Brammar, Berger and Yanofsky (1967) studied phase-shift mutants in the tryptophan synthetase *A* gene of *E. coli*. Two partial revertants, nos. 8 and 11, of mutant 9813 were found to have changes in the amino-acids at positions 174–8 and 173–4 in the molecule, respectively (see Figs. 13.8 and 14.14(*b*)). Nucleotide sequences compatible with the *in vitro* data on the amino-acid code could be assigned to these peptides, and these revealed that mutant 9813 was due to the loss of an adenine nucleotide in codon 174, while the partial revertants 8 and 11 had gained G in codon 178 and A in 173, respectively.

A remarkable feature of these studies on phase-shift mutants, both in *T*4 and its host, is that all the additions of nucleotides are duplications of nucleotides adjacent to the site of the addition (AA, GG and GUGU in place of A, G and GU, respectively).

Crick (1967) examined the published data on the mutational changes known in human haemoglobin and found that the amino-acid substitutions could all be accounted for by single base changes, assuming that the code deciphered with the cell-free system from *E. coli* applied to man. Crick pointed out that all the mutations known at that time in haemoglobin start or finish with charged amino-acids (arginine, aspartic acid, glutamic acid, histidine or lysine) because of the method of detecting the mutants.

It is evident from these studies on mutations in tobacco mosaic virus, *E. coli* and its *T*4 virus, and man, that at least part of the *E. coli in vitro* code applies *in vivo* and to organisms far removed from bacteria in evolution. There is a strong probability, therefore, that at least the greater part of the code will be found to be universal. The first evidence that the amino-acid code was likely to be the same in a wide range of organisms was obtained by von Ehrenstein and Lipmann (1961). They showed that amino-acyl transfer RNA derived from *E. coli*, when mixed with the ribosomes and attached haemoglobin messenger RNA from the reticulocytes of *Oryctolagus*, would bring about the synthesis of haemoglobin. In other words, the bacterial

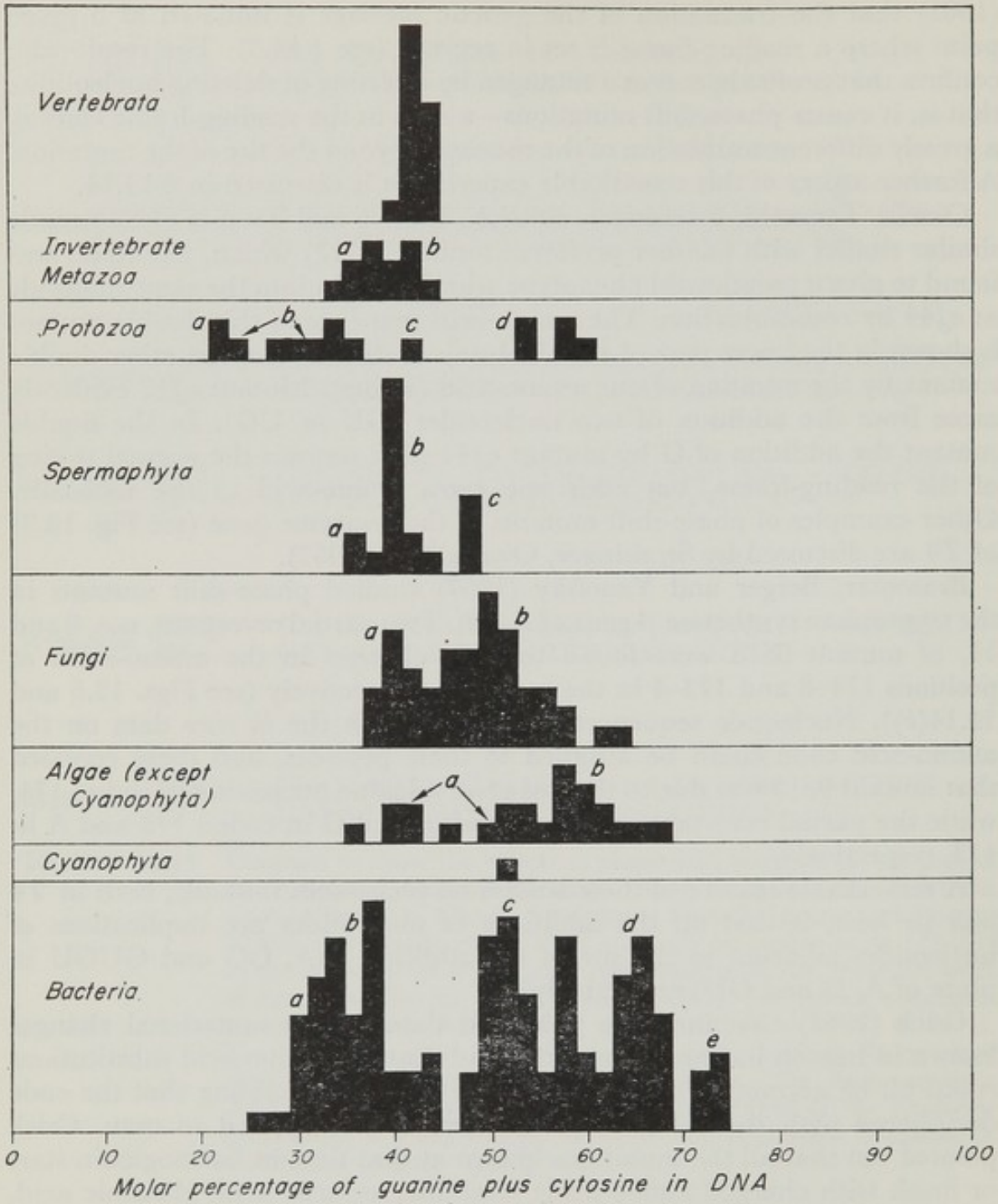


FIGURE 14.15 Histograms to show the molar percentage of guanine plus cytosine out of the total bases (adenine, cytosine, guanine and thymine) in the DNA of 275 organisms from the data tabulated by Sueoka (1964), supplemented with the results given by Jones and Thompson (1963), Tonomura, Malkin and Rabinowitz (1965), Storck (1966), Evans (1966) and Dutta, Richman, Woodward and Mandel (1967). The peaks in the histograms are caused by the following groups of organisms (see Appendix 1 for their classification):

Bacteria: (*a*) Bacillaceae, (*b*) Lactobacteriaceae and Parvobacteriaceae, (*c*) Enterobacteriaceae, (*d*) Achromobacteriaceae and Pseudomonadaceae, (*e*) Actinomycetales.
Algae: (*a*) Colourless Volvocales, Bacillariophyta *et al.*, (*b*) other Chlorophyta *et al.*

transfer RNA was interpreting the mammalian messenger correctly as judged by the fingerprint of the end-product.

That the amino-acid code should include several alternative coding units for most of the amino-acids provides an explanation for an otherwise puzzling phenomenon, namely, the remarkable diversity in the frequency of each base-pair in the DNA of different organisms, particularly among bacteria (cf. § 11.6). The range is from about $\frac{1}{4}$ to $\frac{3}{4}$ of the total. This variation is much greater than could be due to diversity in amino-acid composition of the constituent proteins. The molar percentage of guanine plus cytosine out of the total bases in the DNA of 275 organisms is shown graphically in Fig. 14.15. The uniformity in base composition of the more highly evolved organisms compared with protozoa, fungi, algae and bacteria is apparent. This may reflect the relative length of time that has elapsed since they evolved, or more probably the number of generations, since mutation-rate is related to generation-time (see § 9.5). With alternative codes for the same amino-acid, considerable change in nucleotide composition is possible without affecting the amino-acid composition of the proteins. It is to be expected, therefore, that if a group of organisms has been in existence for a sufficiently long period of time, the nucleotide composition of its DNA will diverge through the occurrence of successive mutations until it spans the permissible range. Figure 14.15 reveals how related organisms have similar DNA compositions; for example, the peaks in the bacterial histogram correspond to particular families (see caption to figure). Conversely, large differences in DNA composition, as between the ciliated and flagellated Protozoa, indicate wide evolutionary divergence.

Sueoka (1961) has shown that part of the variation in DNA base composition in bacteria is to be attributed to alterations in the relative proportions of the various amino-acids in the proteins of the different species. He compared the DNA composition of 11 species with the amino-acid composition of their total protein. The molar proportion of guanine plus cytosine out of the total bases ranged from 35 % to 72 %. Sueoka found that asparagine and aspartic acid (which were not distinguished from one another), glutamine and glutamic acid (also not distinguished), isoleucine, lysine, phenylalanine and tyrosine were relatively more abundant in the species with a low proportion of guanine and cytosine than in those with a high content of this base-pair. Reference to Table 14.7 shows that these amino-acids (except for glutamine and glutamic acid) have a relatively low guanine–cytosine content

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Fungi: (*a*) Mucorales and Endomycetales, (*b*) Eu-Ascomycetes (including Fungi Imperfecti) and Basidiomycetes.
Spermaphyta: (*a*) Liliaceae, (*b*) Dicotyledones and 1 conifer, (*c*) Gramineae.
Protozoa: (*a*) Acrasiales, (*b*) Ciliata (Holotricha), (*c*) Myxomycetales, (*d*) Flagellata (Zoomastigina).
Invertebrate Metazoa: (*a*) Echinodermata and 1 mollusc, (*b*) Crustacea, 1 annelid and 1 orthopteran.
Vertebrata: These consist of 4 fishes, 1 amphibian, 2 reptiles, 1 bird and 9 mammals (including man with 40% guanine + cytosine).

for their coding nucleotides. Conversely, Sueoka found that alanine, arginine, glycine and proline were more abundant in species with a high proportion of this base-pair and from the table it is evident that these amino-acids are coded by nucleotides with a high guanine-cytosine content. The remaining amino-acids (apart from cysteine and tryptophan which were not estimated) showed no regular variation in frequency with DNA base content, and from the table it is evident that, except for leucine and methionine, they are coded by triplets which in the aggregate contain equal numbers of each base-pair. This comparison of amino-acid and nucleotide frequencies clearly shows that some of the diversity in DNA composition is related to diversity in protein composition. It also suggests that the code worked out for *E. coli in vitro* is similar if not identical for a range of bacteria *in vivo*.

Fitch (1967) surveyed 50 mutational changes in human haemoglobin and 229 in cytochrome *c* of various organisms. He assumed that the mutant forms of haemoglobin (cf. §§ 13.5 and 13.7) have been derived from the normal and that the diversity in the primary structure of the cytochrome *c* of 3 fungi, 2 insects, 1 fish, 2 reptiles, 4 birds and 8 mammals has evolved from a hypothetical ancestral molecule. For both proteins, applying the *E. coli* code, he found a marked excess of the change from guanine to adenine in the messenger compared with all the other 11 possible base substitutions including the complementary transition (cytosine to uracil). Another unexplained anomaly—the shortage of the *CpG* doublet in the DNA of chromosomal organisms—was mentioned in § 12.5. Subak-Sharpe *et al.* (1967) have inferred that in mammals (where *CpG* is particularly uncommon) selection may have acted to eliminate this doublet from within codons. This is possible because the amino-acids coded by triplets containing the sequence *CG* (that is, serine, proline, threonine, alanine and arginine—see Table 14.7) are also coded by triplets lacking this doublet. Fitch's conclusions and those of Subak-Sharpe *et al.* imply that, for an unknown reason, selection has favoured specific mutational changes in the particular DNA chain which is transcribed into messenger RNA.

§ 14.14 The direction of translation (reading of message)

Thach, Cecere, Sundararajan and Doty (1965) established the direction in which messenger RNA is translated into amino-acid sequence. A hexanucleotide of composition AAAUUU (5′-phosphate terminus to the left, 3′-hydroxyl terminus to the right) was used in the cell-free system of protein synthesis and found to generate a dipeptide of composition NH_2-lysine-phenylalanine-COOH. Since polypeptide synthesis is known to begin at the amino end (see § 14.10) and since AAA is known to code for lysine and UUU for phenylalanine, it was inferred that the messenger is read in the 5′ to 3′ direction. Salas, Smith, Stanley, Wahba and Ochoa (1965) independently obtained a similar result. Using the cell-free protein synthesizing system they found that polynucleotides of structure (5′)AAA . . . AAC(3′), with 21 to 23 nucleotide residues, directed the synthesis of NH_2-lysine-lysine- . . . -lysine-asparagine-COOH, from which they inferred that the message is read from the 5′- to the 3′-end of the polynucleotide.

Yanofsky and associates established the co-linearity of gene and polypeptide using the *A* protein of tryptophan synthetase in *Escherichia coli* and the corresponding gene (§ 13.8). They found that mutants *A*23 and *A*46 had the amino-acids shown in Table 14.8 at position 210 in the polypeptide and gave wild-type recombinants when crossed (§ 14.13). These and other changes in this codon due to mutation and recombination (see Fig. 14.13)

TABLE 14.8 Data of Yanofsky, Ito and Horn (1967) for position 210 in the *A* protein of tryptophan synthetase in *Escherichia coli*. The codons are inferred from the more extensive changes given in Fig. 14.13.

Strain	Amino-acid at Position 210	Codon
*A*23	arginine	5′ AGA 3′
wild-type	glycine	5′ GGA 3′
*A*46	glutamic acid	5′ GAA 3′

were compatible with the codons given in Table 14.8. Guest and Yanofsky (1966) crossed *A*23 with *A*46 in conjunction with other linked mutants and found from the behaviour of the linked mutants that the site of *A*23 was the nearer of the two to the end of the gene corresponding to the amino-terminal end of the polypeptide. From its codon *A*23 is evidently also the nearer of the two to the 5′ end of the messenger. Thus the 5′ to 3′ orientation of the messenger corresponds to the amino to carboxyl orientation of the polypeptide.

Terzaghi *et al.* (1966) in their study of phase-shift mutants in virus *T*4 of *E. coli* (§ 14.13) found that codons compatible with the two sequences of amino-acids (Fig. 14.14) could be obtained only if the triplets were orientated with the 5′ to 3′ polarity corresponding to the N-terminal to C-terminal polarity of the amino-acids. Since it had been shown that polypeptide synthesis proceeds from the N-terminal to the C-terminal end, the messenger must be translated from the 5′ to the 3′ end.

Thus, these results, obtained by various means, are in agreement with one another, and establish that translation takes place in the same direction as transcription (§ 14.8). The possibility thus arises that translation may begin before messenger synthesis has been completed, or even that the two processes are coupled in some way. This is discussed in § 15.3 and § 15.4.

§ 14.15 The wobble theory

Crick (1966) considered the possible base-pairing between codons on messenger RNA and anticodons on transfer RNA. He noted that in the first two positions of the codon (see Table 14.7) the four bases were clearly distinguished by the anticodon, and he inferred that the pairings in these two positions were the standard ones. Owing to the antiparallel direction of the chains, bases 1 and 2 in the codon will pair with bases 3 and 2 respectively in the anticodon (see Figs. 14.9 and 14.11).

The multiple coding for the same amino-acid is due chiefly to variation in the third position in the codon (Table 14.7), suggesting that at this position

one transfer RNA molecule might recognize more than one nucleotide owing to alternative possibilities for base-pairing. Crick argued that, if the first two bases in the codon paired in the standard way, the pairing in the third position might be close to the standard ones. He assumed that base no. 3 in the codon is always paired with the anticodon and furthermore that at this position, as Table 14.7 shows, the pyrimidines (U and C) can sometimes be distinguished from the purines (A and G), for example, in coding for

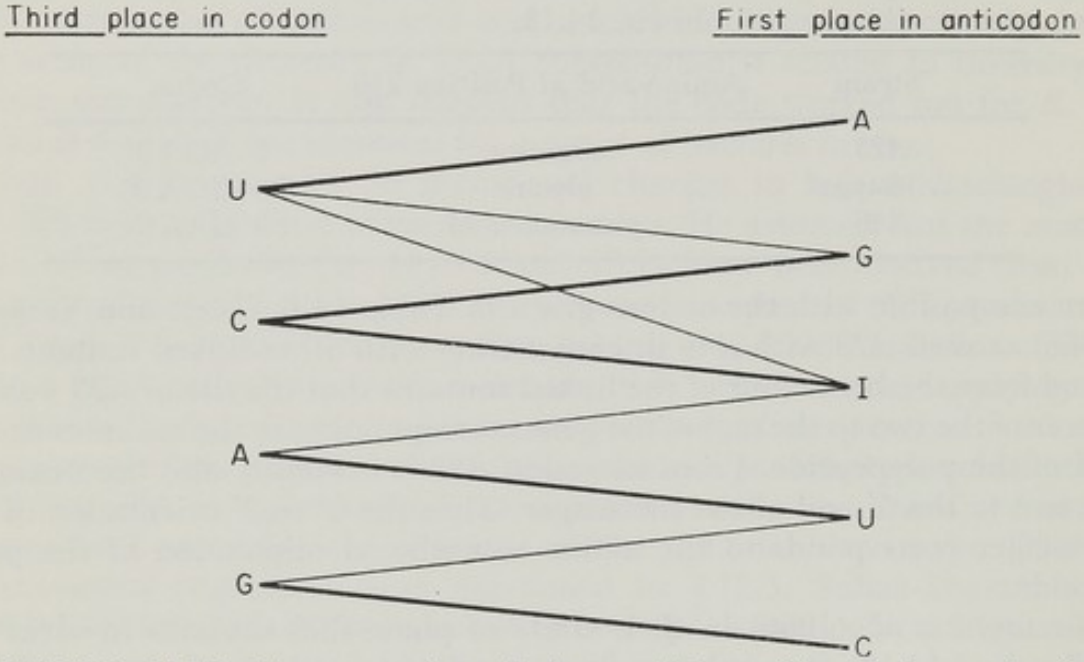


FIGURE 14.16 Diagram to show the bases which can form hydrogen bonds between the third place in the codon in messenger RNA and the first place in the anticodon in transfer RNA according to the wobble hypothesis of Crick (1966). The letters A, C, G, I and U stand for the nucleosides adenosine, cytidine, guanosine, inosine and uridine, respectively, corresponding to the bases adenine, cytosine, guanine, hypoxanthine and uracil. Heavy lines show the standard base pairs and the thinner lines the extra pairs permitted.

histidine and glutamine. From this he deduced that, among the various possible abnormal base-pairs, pairing between one pyrimidine and another did not occur. He considered that the likely rules for pairing were as follows. In addition to the standard pairs (which include cytosine in the codon pairing with hypoxanthine in the anticodon) slight wobble allows (a) uracil in the codon to pair with guanine or hypoxanthine in the anticodon, (b) adenine in the codon to pair with hypoxanthine in the anticodon, and (c) guanine in the codon to pair with uracil in the anticodon (see Fig. 14.16). There is support for the pairing of guanine with uracil from the data on nucleotide sequence in transfer RNAs and 5*S* ribosomal RNA, where single G-U pairs have been found in otherwise normally base-paired regions.

The wobble hypothesis predicts that an amino-acyl transfer RNA which recognises a triplet ending in *C* will also recognise at least one other triplet. A similar prediction applies to a triplet ending in *A*. The data in Table 14.7

agree with these predictions, for there are no amino-acids corresponding to such triplets alone. The hypothesis also predicts that when an amino-acid (such as valine) is coded by all 4 bases in the third position, there must be at least 2 transfer RNAs for this amino-acid. These would have either G and U respectively at the first position in the anticodon (corresponding to U/C and A/G in the codon), or I (inosine—the nucleoside of hypoxanthine) and C respectively (corresponding to U/C/A and G in the codon).

The anticodons of 8 transfer RNAs are given in Table 14.9, together with the codons predicted by the wobble hypothesis. These are all in agreement

TABLE 14.9 The table shows the anticodons of 8 transfer RNAs, and the codons with which they are predicted to pair on the wobble hypothesis. The anticodons are derived from the results of the authors shown in the legend to Fig. 14.9. 2′-O-Methylguanosine at position 1 in the anticodon is expected to pair like guanosine at that position. Pseudouridine (Ψ) at position 2 is expected to pair like uridine at that position.

Species and Amino-acid		Anticodon	Codons Predicted
Saccharomyces cerevisiae	Alanine	IGC	GCU, GCC, GCA
	Serine	IGA	UCU, UCC, UCA
	Valine	IAC	GUU, GUC, GUA
	Phenylalanine	G[1]AA	UUU, UUC
	Tyrosine	GΨA	UAU, UAC
Escherichia coli	Tyrosine	G[2]UA	UAU, UAC
	Tyrosine[3]	CUA	UAG
	Formyl-methionine	CAU[4]	AUG, G[4]UG

[1] 2′-O-Methylguanosine.
[2] Unidentified guanosine derivative.
[3] Amber suppressor (see § 14.17).
[4] Wobble at third position in anticodon (see § 14.16).

with the code established for *Escherichia coli in vitro*. Moreover, Söll, Cherayil and Bock (1967) have tested the binding of amino-acyl transfer RNAs to ribosomes in the presence of trinucleotides known to be codons for the respective amino-acids, and have found that particular *t*RNAs can often recognize more than one codon differing in the third base. Furthermore, the multiple recognition patterns were those predicted by the wobble hypothesis: for example, the major serine *t*RNA in *Saccharomyces cerevisiae* showed binding with the trinucleotides UCU, UCC and UCA, but not with the other serine codons (UCG, AGU and AGC). This is what was expected on wobble theory from the known anticodon (see Table 14.9).

Convincing evidence for the multiple recognition by one kind of transfer RNA of codons differing in the third base was obtained by Söll and RajBhandary (1967). Their technique was to synthesize a polypeptide in the cell-free system using ribopolynucleotides of known repeating sequence as messengers and with purified amino-acyl *t*RNA of one particular kind. They found, for example, that phenylalanyl-*t*RNA from *S. cerevisiae* translates

the codons UUU and UUC with equal efficiency, as predicted from its anticodon (see Table 14.9).

Söll, Cherayil and Bock (1967) found that, although the amino-acid code is apparently the same in *S. cerevisiae* and *E. coli*, the patterns of codon recognition by transfer RNAs show some differences. Thus, in *E. coli* one valine *t*RNA recognizes GUU and GUC and another GUA and GUG, while in *S. cerevisiae* the major valine *t*RNA recognizes GUU, GUC and GUA (Table 14.9). A similar difference is shown by the serine and alanine *t*RNAs. Such results were expected because the *t*RNAs of *S. cerevisiae* were known to be richer in inosine than those of *E. coli*. (Inosine is the nucleoside needed in the first place of the anticodon for the recognition of U, C and A in the third place of the codon—see Fig. 14.16.) These differences, however, between the anticodons of *S. cerevisiae* and *E. coli* are not found for every amino-acid that is coded by all 4 bases in the third position. Thus, in both organisms arginine shows the 3 and 1 pattern (one *t*RNA recognizing U, C and A in the third position and the other recognizing G) and glycine the 2 and 2 pattern (U and C *versus* A and G). With glycine in *S. cerevisiae*, however, a third *t*RNA is known which recognizes GGG alone.

Multiplicity of transfer RNAs for the same amino-acid is known of yet another kind: Holley *et al.* (1965) found that at position *n*1 in alanine *t*RNA of *S. cerevisiae* there was variability, this base being either uracil or dihydrouracil. Zachau *et al.* (1966) found two forms, I and II, of serine *t*RNA in *S. cerevisiae*, which, unlike the previous example, would require to be coded by different genes: they had the same anticodon but differed in the bases at *l*2, *p*4 and *p*6. Likewise, Goodman *et al.* (1968) found there were two forms of *t*RNA for tyrosine in *E. coli* which differed in the bases at positions *l*2 and *l*3, and Dube *et al.* (1968) found variability at *j*3 in formyl-methionine *t*RNA. Söll and associates also found two *t*RNAs in *E. coli* for each of the codon pairs UUU and UUC (phenylalanine), AUU and AUC (isoleucine), and AAA and AAG (lysine). They suggested that the multiplicity of equivalent *t*RNAs could be of great biological significance as a safeguard against the often lethal effect of a mutation in a *t*RNA gene. This multiplicity also explains the origin of suppressor mutations.

Brody and Yanofsky (1963) found that a suppressor gene can alter the primary structure of a protein. Mutant *A*36 leads to the insertion of arginine instead of glycine at position 210 (Fig. 13.8) in the *A* protein of tryptophan synthetase in *E. coli*. This makes the enzyme non-functional and hence imposes a requirement for tryptophan on the bacterium. A mutation of a suppressor gene, which was not linked to the *A* gene, caused mutant *A*36 to become wild-type in phenotype, and it was found that glycine was inserted again in place of the arginine. Brody and Yanofsky suggested that the suppressor gene probably interfered with the translation of the *A* gene messenger RNA into polypeptide by altering the specificity of the glycine or arginine activating enzymes or transfer RNAs.

A special class of suppressor mutations in which the mutant suppressed fails to synthesize a complete polypeptide is discussed in § 14.17. It has now been established that suppression of such mutants can arise through the

occurrence of mutation in a gene for a transfer RNA at the position of the anticodon, a duplicate unmutated gene also being present.

The occurrence of more than one transfer RNA for the same amino-acid, in addition to its safeguard against lethal mutations, may have been favoured for another reason. Variation in the primary structure of the α chain of haemoglobin is known in several mammals (*Mus musculus*, Rifkin, Rifkin and Konigsberg, 1966; *Oryctolagus cuniculus*, von Ehrenstein, 1967; *Equus caballus*, Kilmartin and Clegg, 1967). Heterozygosity can be ruled out as an explanation, at least in *Mus*, because the mice had been inbred for 82 generations. Von Ehrenstein (1967) suggested ambiguous translation of the messenger RNA as an explanation. In several instances he found an association between ambiguity and minor *t*RNAs. Thus, at position 48 in the α chain of *Oryctolagus*, where leucine or phenylalanine may occur, the codon could be served, as far as leucine was concerned, only by a minor *t*RNA which did not react with any other leucine codon in either the α or β chains. He suggested that at positions such as this two different minor *t*RNAs carrying different amino-acids recognize the same codon. The minor *t*RNAs would thus allow ambiguity of translation at specific points in the polypeptide determined by rare codons. The resulting diversity within individuals in the primary structure of the protein can evidently be advantageous, to judge by its widespread occurrence in haemoglobin. Von Ehrenstein pointed out that the ambiguity of translation, if confirmed, will mean that the theory of one gene: one polypeptide is more correctly stated as one gene: one family of related polypeptides.

Fuller and Hodgson (1967) constructed three-dimensional models of transfer RNA. On the basis of the known nucleotide sequences, and evidence from X-ray diffraction on the conformation of base-paired regions, they derived a model for the anticodon arm. Earlier X-ray diffraction studies (Arnott *et al.* 1966) of double helical RNA from reovirus and other sources had suggested that the base-pairs may be tilted 17° instead of being set perpendicular to the helix axis as in DNA. The tilting would allow 11 nucleotide-pairs per turn of the helix in 2·8 mμ, compared with 10 pairs in 3·4 mμ in DNA. Fuller and Hodgson assumed that the base-paired region of the anticodon arm (segments *g* and *i* in Fig. 14.9) has this 11-fold double helical structure. In their model, 5 of the 7 nucleotides of the anticodon loop (*h*3–*h*7 in Fig. 14.9) are stacked in this 11-fold conformation so that they lie on the same helix as the nucleotides in the adjoining double helical region (segment *i*). This model represents a unique solution to the problem of maximizing base-stacking in the anticodon loop, and moreover it accounts for the wobble in pairing at position no. 1 in the anticodon, because this is the last nucleotide (*h*3) of the 5 in the stack and the helix begins to become distorted at this point for the return loop (nucleotides *h*2 and *h*1). The alternative pairing at position 3 in the codon would thus arise through distortion of the anticodon and not of the codon.

It is evident that Crick's wobble theory is not only supported by experimental evidence from the use of specific amino-acyl transfer RNAs in the systems used to establish the amino-acid code, but it is also in keeping with

a likely three-dimensional structure for the anticodon loop. The theory has been extended to account for variability at the first position in the codon in the special case of the transfer RNA responsible for initiating polypeptides (see § 14.16).

§ 14.16 Polypeptide initiation

Marcker and Sanger (1964) discovered that in *Escherichia coli* and *Saccharomyces cerevisiae*, and also in cell-free systems derived from *E. coli*, formylation of the α amino group of methionine ($NH_2{\cdot}CH(CH_2{\cdot}CH_2{\cdot}SCH_3)CO{\cdot}OH$) can take place, and that this occurs after the methionine has become attached to its transfer RNA, thus giving rise to N-formyl-methionyl transfer RNA ($OHC{\cdot}NH{\cdot}CH(CH_2{\cdot}CH_2{\cdot}SCH_3)CO{\cdot}O{\cdot}$*t*RNA). No other amino-acid showed formylation. Marcker (1965) established that the formylation was catalysed by a specific enzyme, a transformylase, and he suggested, among various possibilities, that formyl-methionyl-*t*RNA could function as a polypeptide initiator.

This hypothesis was confirmed by Adams and Capecchi (1966), who labelled formyl-methionyl-*t*RNA with ^3H in the formyl group, and studied the incorporation of the label into proteins synthesized with the *E. coli* cell-free system, using the RNA of virus *R*17 of *E. coli* as messenger. They found that the labelled formyl groups were incorporated into at least two of the 3 proteins coded by *R*17 RNA. Since it seemed likely that the formylation prevented the amino group of the methionine from taking part in peptide bond formation, the formylated methionine could be incorporated into a polypeptide only as the N-terminal amino-acid. Adams and Capecchi concluded that formyl-methionyl-*t*RNA was the initiator of protein synthesis. They were puzzled, however, because the N-terminal amino-acid of the coat protein of the intact virus particle is alanine. The coat protein made *in vitro* was digested with pronase and this resolved the paradox: they found that although all the labelled formyl groups were in N-formyl-methionine, the amino-acid next to this was alanine. They concluded that *in vivo* the initial formyl-methionine of the coat protein is removed enzymatically, yielding protein with N-terminal alanine. Webster, Engelhardt and Zinder (1966) obtained similar results using the RNA of virus *f*2 as messenger.

Clark and Marcker (1966) showed there were two methionine transfer RNAs in *E. coli*. They separated them by counter-current distribution and found that none of the attached methionine of the faster moving one (met-$tRNA_1$) could be formylated, whereas all that of the slower (met-$tRNA_2$) could be. Using the cell-free system, they found that the $tRNA_2$, whether carrying methionine or formyl-methionine, could initiate polypeptides, whereas met-$tRNA_1$ inserted methionine only in internal positions in a polypeptide. It was evident that the specificity of the $tRNA_2$ as a polypeptide initiator resided in the structure of this transfer RNA and not in the formyl group. Clark and Marcker found that the internal met-*t*RNA (that is, no. 1) would bind only to trinucleotides of sequence AUG, while the initiating met-*t*RNA (no. 2) bound to GUG as well as AUG. Clark *et al.* (1968) found that a fragment from *g*1 to *j*2 (Fig. 14.19) of the initiating met-*t*RNA

specifically bound to ribosomes in the presence of AUG or GUG. This was the first direct evidence for the position of the anticodon in any *t*RNA.

Bretscher and Marcker (1966) obtained evidence that the initiating met-*t*RNA binds to the peptide site (see § 14.10 and Fig. 14.17) of the ribosome, while the internal met-*t*RNA binds in the normal way to the amino-acid site. The antibiotic puromycin is believed to inhibit protein synthesis by substituting for an amino-acyl-*t*RNA and reacting with peptidyl-*t*RNA bound to the peptide site of the ribosome. Bretscher and Marcker found that the internal met-*t*RNA, when bound to ribosomes with the triplet AUG, was comparatively insensitive to puromycin, whereas the initiating met-*t*RNA when so bound was found to be sensitive to the antibiotic; this sensitivity was manifest whether the methionine was formylated or not.

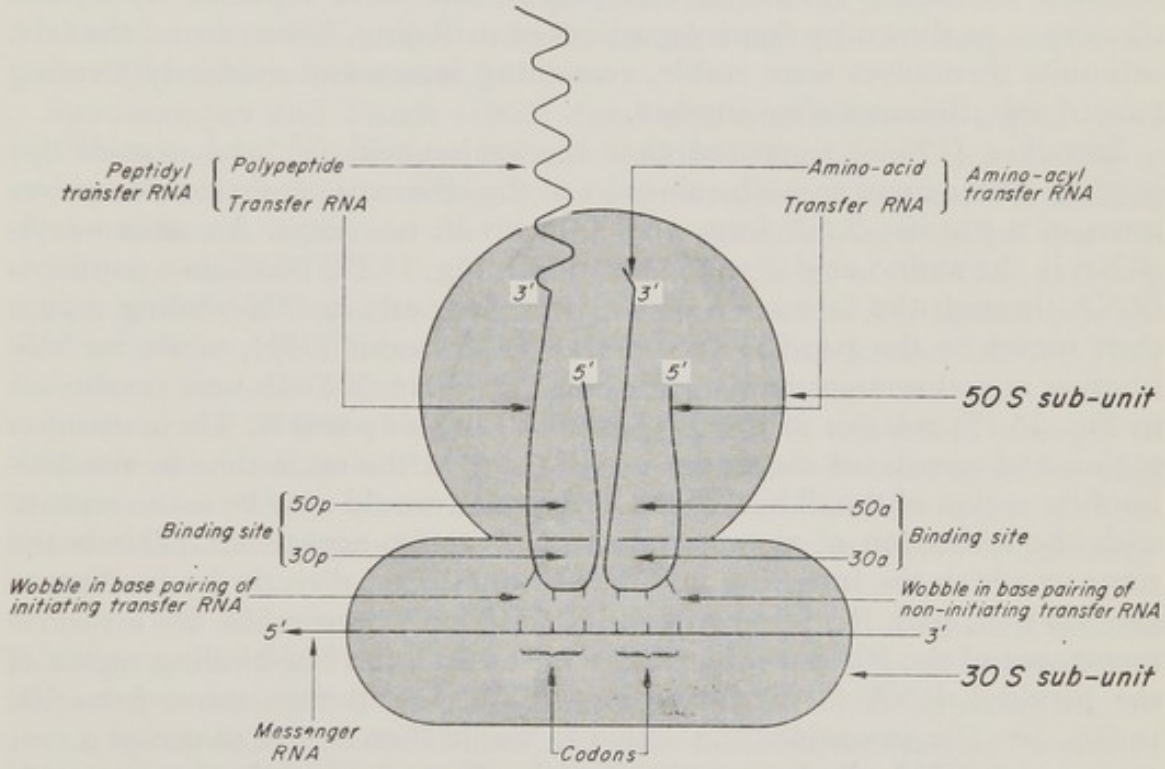


FIGURE 14.17 The diagram is based on that of Bretscher and Marcker (1966) and shows a ribosome during protein synthesis. The binding-sites for amino-acyl and peptidyl transfer RNAs on the ribosomal sub-units are indicated by *a* (amino-acid) and *p* (peptide).

Nomura and Lowry (1967) discovered that in the presence of the RNA from virus *f*2 of *E. coli* as messenger the initiating met-*t*RNA will bind to the 30*S* ribosomal sub-unit but not to the intact 70*S* ribosome. No other amino-acyl-*t*RNA showed this effect. They proposed that the first step in protein synthesis is the formation of an initiation complex consisting of the 30*S* particle, messenger RNA with the appropriate codon, and formyl-methionyl-*t*RNA. They obtained evidence that the other sub-unit—the 50*S* particle—then joins the initiation complex and allows amino-acyl-*t*RNAs other than the initiating met-*t*RNA to bind with the ribosome. Direct support for this hypothesis was obtained by Ghosh and Khorana (1967) using synthetic messengers containing AUG or GUG: they found that the

30*S* sub-unit binds the initiating met-*t*RNA in the presence of one of these codons, and then the joining of the 50*S* sub-unit to the 30*S* forms a second site which now binds non-initiating amino-acyl-*t*RNAs (Fig. 14.17).

Mangiarotti and Schlessinger (1966), from study of ribosome metabolism, concluded that the 30*S* and 50*S* sub-units join together only in association with protein synthesis and that these particles separate and return to a pool of sub-units when the polypeptide synthesis has been completed. This model of ribosome behaviour is in keeping with Nomura and Lowry's hypothesis of how protein synthesis is initiated. Support for Mangiarotti and Schlessinger's idea was obtained by Kaempfer, Meselson and Raskas (1968). Bacteria labelled with heavy isotopes (^{13}C, D, ^{15}N) were transferred to a medium containing the normal isotopes. During growth in this light medium the heavy ribosomes disappeared and were replaced by hybrid ribosomes, as shown by density-gradient centrifuging. It was found that the sub-units themselves were stable, remaining intact and evidently forming part of one ribosome after another.

Bretscher (1968*b*) suggested that the amino-acid (*a*) and peptide (*p*) binding sites extend to both sub-units of the ribosome, and that the movement of a transfer RNA from *a* to *p* occurs in two steps. An amino-acyl-*t*RNA in the amino-acid site (30*a* and 50*a* in Fig. 14.17) becomes a peptidyl-*t*RNA through the formation of the peptide bond; its 30*S*-binding region then moves to the peptide site on the 30*S* sub-unit (30*p*), while its 50*S*-binding region remains in 50*a*; displacement of the 50*S* sub-unit (to the left in Fig. 14.17) relative to the 30*S* would make this possible. The anticodon region and associated messenger would move at the same time as the 30*S*-binding region of the *t*RNA, so the next codon would now be set to register with the anticodon of an appropriate new amino-acyl-*t*RNA. This is the stage to which the initiating met-*t*RNA would lead directly by binding to site 30*p* and, with the joining of the 50*S* sub-unit, to site 50*a*. By a reverse movement of the 50*S* particle relative to the 30*S*, the 50*S*-binding region of the peptidyl-tRNA (or initiating met-*t*RNA) would then move from 50*a* to 50*p*. Site 50*a*, in conjunction with 30*a*, would then be free to accept a new amino-acyl-*t*RNA, in preparation for the formation of the next peptide bond. According to Dintzis (1961) the synthesis of a haemoglobin polypeptide of about 150 amino-acids takes 1½ minutes at 37°, so the entire cycle of events between the formation of one peptide bond and the next would occupy just over half a second.

In view of the binding of the initiating met-*t*RNA with the triplet GUG as well as AUG, Bretscher and Marcker (1966) suggested that when a *t*RNA is in the peptide site of the ribosome the first base in the codon may not be specifically recognised, in contrast to *t*RNA in the amino-acid site, where it is the third base which shows the wobble in pairing (see § 14.15 and Fig. 14.17). Support for this inverted wobble was obtained by Dube, Marcker, Clark and Cory (1968). They established the complete nucleotide sequence of the initiating *t*RNA of *E. coli*, using the technique of Sanger, Brownlee and Barrell (see § 14.11), and found that the anticodon was CAU. Assuming that for this *t*RNA the wobble theory applies to the third base of the anticodon, the occurrence of uracil at this position means the codons predicted are AUG

and GUG (see Fig. 14.16 and Table 14.9), in agreement with observation.

The lengths of the various segments of the initiating *t*RNA were found by Dube *et al.* to be as shown in Table 14.4 (column headed f.met.), and the identity and positions of minor nucleosides to be as shown in Table 14.5. The nucleotide composition and sequence do not reveal, however, which part is responsible for the binding to the peptide site, nor where the specificities for met-*t*RNA synthetase and transformylase lie. A peculiarity of the molecule is that the nucleotide at the 5′ terminus (*a*1 in Fig. 14.9) is not paired, but this is not necessarily of special significance: the *t*RNA for alanine in *S. cerevisiae* shows no pairing at position *a*6. Another peculiarity of the initiating *t*RNA of *E. coli* is the presence of adenine instead of a minor purine on the 3′ side of the anticodon, but this feature is shared with valine *t*RNA of *S. cerevisiae*. Dube *et al.* hope that study of the nucleotide sequence of the other met-*t*RNA of *E. coli* will reveal what sites are responsible for their differences in behaviour.*

Sundararajan and Thach (1966) discovered that when the triplet AUG was incorporated into longer polynucleotides it suppressed the reading of codons which partially overlapped its sequence, and promoted the reading of the adjacent codon on the 3′ side. For example, they found that the sequence AUGUUUU . . . stimulated the binding of *t*RNAs carrying methionine (and therefore registering with the codon AUG) and phenylalanine (codon UUU), but not those carrying valine (codon GUU). They also found that AUG had this ability to fix the reading-frame even when it was not at the 5′ end of the chain. Thus, AAAUG was found strongly to stimulate the binding of *t*RNAs carrying methionine but not those carrying lysine (codon AAA), whereas AAACG coded very well for lysine. It thus appears that the triplet AUG determines the phase in which the message is read.

Smith and Marcker (1968) have found that N-formyl-methionyl transfer RNA occurs in association with the 70*S* ribosomes of the mitochondria of *Saccharomyces* and *Rattus*, but not in association with the 80*S* ribosomes present elsewhere in the cytoplasm of these organisms. Smith and Marcker conclude that another mechanism of polypeptide initiation exists on 80*S* ribosomes.

§ 14.17 Polypeptide termination

Benzer and Champe (1962) devised an ingenious technique for recognising mutants which blocked the synthesis of a polypeptide. Mutant *r*1589 in the *r*II region of virus *T*4 of *E. coli* is a deletion that includes the junction of the *A* and *B* genes, and part of each (see § 14.3). The *A* function is thereby lost, but the *B* function remains: this was shown by the fact that *r*1589 will complement with any *r*II *B* mutant that has an intact *A* gene, thereby allowing growth on strain *K* of the host. (Both *A* and *B* activities are needed for such growth). Evidently the lost end of the *B* gene is non-essential. Crick *et al.* (1961) showed, however, that the *B* function can be turned off

* This hope has not been realised: Cory *et al.* (1968) found that they differ in 39 of their 77 nucleotides, with differences in all parts of the molecule.

by transferring (through recombination) certain deletions and proflavin-induced mutants into the *A* fragment (see Fig. 14.3(*p*) and (*q*)). They suggested that in *r*1589 the *A* and *B* fragments are transcribed into a single messenger RNA, which is then translated into a single polypeptide. A phase-shift mutation in the *A* gene would then affect the *B* gene as well, and likewise with a mutant that interrupted polypeptide formation.

Benzer and Champe applied this test for polypeptide-terminating mutations in the *A* gene to mutants described as ambivalent because they were inactive in one strain of *E. coli* but active in another. The host strain which suppressed the viral mutants had arisen by mutation. The viral mutants reverted on treatment with 2-aminopurine, and were therefore thought to have arisen by base substitution and not by addition or deletion of nucleotides (§ 14.2). Five such mutants, when combined with *r*1589 as a double mutant, showed no *B* gene activity. It was concluded that they blocked polypeptide synthesis. All were suppressed by the same mutant strain of the host. It was inferred that when the host contained a suppressor, the coding unit from the virus would be translated as an amino-acid, thereby permitting protein synthesis to continue. This misreading implied that the suppressor mutation affected the translation mechanism, most probably modifying either a transfer RNA or its activating enzyme. A likely possibility was that the suppressor mutation affected the fit of a transfer RNA to a coding unit. As indicated below, this anticodon hypothesis has now been confirmed for one suppressor.

Garen and Siddiqi (1962) likewise discovered mutants which apparently led to premature stopping of polypeptide synthesis, and the effect of which was suppressed by mutation of another gene. The suppressible mutants were in the gene for alkaline phosphatase in *E. coli* and the polypeptide termination was deduced from the lack of any enzyme protein in these mutants. Benzer and Champe found that the suppressor of the phosphatase-deficient mutants also suppressed one group of the ambivalent *r*II mutants which they had studied.

Confirmation that in ambivalent ('amber') mutants of *E. coli* polypeptide synthesis ends prematurely was obtained from study of the effect of such mutants on the protein of the head of virus *T*4 (see § 13.8).

Weigert and Garen (1965) studied the reversion of an amber mutant of the alkaline phosphatase gene of *E. coli*. The amino-acid at the position in the polypeptide corresponding to its premature end in the mutant was identified in 21 revertants, and from the range of amino-acids found and the known codon assignments for them, it was inferred that there was an RNA triplet indicating the end of the polypeptide, and that this triplet was UAG.

Brenner, Stretton and Kaplan (1965) also found that the RNA nucleotide triplet coding for polypeptide termination in amber mutants was UAG. They used mutants of virus *T*4 in which the synthesis of the protein of its head was stopped prematurely by such mutants and they identified the amino-acids which occurred at these points when the virus was grown on a mutant bacterial strain (an amber suppressor) which allowed the protein synthesis to go to completion. From the codons assigned to these amino-acids

they deduced the codon for polypeptide-end, since only single nucleotide change would be expected to distinguish it from each of these amino-acid codons. This was the same argument as Weigert and Garen had used.

Brenner, Stretton and Kaplan found that in another group of mutants of virus *T*4 called 'ochre' mutants, the mutant character of which was suppressed by different strains of *E. coli* from those which suppressed amber mutants, the RNA codon for the end of the polypeptide was UAA. The suppressors of ochre mutants were found to be too weak to allow the isolation of ochre mutants of the protein of the head of the virus. The method that had been used to identify the amber triplet could not therefore be applied to the ochre mutants, nor could the hypothesis that ochre mutants result in polypeptide termination be tested. That UAA was the ochre triplet was shown, however, by study of the production and reversion of *r*II ochre and amber mutants using chemical mutagens, in conjunction with the observation that ochre mutants can be converted into amber mutants by mutation. Brenner and Beckwith (1965) found that, although the *E. coli* strains in which amber mutants were suppressed failed to restore *r*II activity to ochre mutants, the converse was not true: strains suppressing ochre mutants also suppressed amber mutants. They inferred that the ochre suppressors recognize both the ochre (UAA) and amber (UAG) triplets, whereas amber suppressors recognise UAG only.

Brenner and Stretton (1965) obtained evidence that amber and ochre mutants cause polypeptide termination not by producing a shortened messenger RNA in transcription from DNA but in the translation from RNA to polypeptide. They studied the effects of phase-shift mutants in the *r*II region of virus *T*4 in conjunction with amber or ochre mutants. Strains were obtained in which an amber or ochre mutant was situated between a plus and a minus phase-shift mutant. The amber and ochre mutants had no effect when so placed: they did not cause polypeptide termination, nor did they affect the reading of the remainder of the gene. It was inferred that amber and ochre mutants were base substitutions sensitive to the phase of reading the message, and that they were recognised in protein synthesis and not in messenger RNA formation.

Using the cell-free protein synthesizing system derived from *E. coli*, Morgan, Wells and Khorana (1966) obtained negative evidence that the triplet UGA, like UAA and UAG, does not code for any amino-acid. By the techniques described in § 14.12 they obtained a synthetic RNA with the repeating sequence GAUGAUGAU . . . . Despite the phasing effect of AUG (see § 14.16) this messenger induced the production of a polypeptide containing aspartic acid, corresponding to the frame set to read GAU, as well as a methionine polypeptide corresponding to AUG, but there was no polypeptide corresponding to UGA.

Brenner, Barnett, Katz and Crick (1967) confirmed from genetical studies that the triplet UGA did not stand for any amino-acid. They found that a particular mutant induced by 2-aminopurine and situated near the left-hand end of the *B* gene of the *r*II region of virus *T*4 (cf. Table 14.1) was not suppressed by any amber or ochre suppressor and was therefore not UAG

or UAA. The mutant was converted to an ochre mutant by hydroxylamine, and the ochre triplet so produced did not require any replication for expression. This suggested that a change from G to A had occurred in the messenger (cf. § 14.2), and, as the mutant was not an amber, its sequence was evidently UGA. Brenner *et al.* argued that it was unlikely that this polypeptide-terminating sequence functioned in transcription from DNA to RNA rather than in translation from RNA to protein because, like other terminating codons, the effects of UGA depended on its being read in phase: when placed in a shifted frame (between a plus and a minus phase-shift mutant) its effect vanished.

Sambrook, Fan and Brenner (1967), following treatment of *E. coli* with a chemical mutagen, isolated a host mutant which specifically suppressed UGA mutants in the *r*II *B* gene of virus *T*4. The suppressor was found to have no effect on ochre or amber mutants at the same sites. This enabled the authors to use the UGA suppressor to select for mutation from ochre (UAA) to UGA in the β-galactosidase gene of *E. coli*. It was known that UAA and UAG mutants of this gene show a polar effect which seems to be a consequence of polypeptide termination: see § 15.3. UGA mutants of the galactosidase gene were found also to be polar. Amber and ochre suppressors failed to restore the galactosidase activity destroyed by the UGA mutants. Sambrook *et al.* concluded that UGA, like UAA and UAG, results in polypeptide termination. In view of the lack of strong suppressors for UAA (in contrast to the other two triplets), they suggested that UAA was the triplet commonly used by *E. coli* to signal the end of a polypeptide.

The ability of amber suppressors to suppress mutations in more than one gene implies that they probably act at one of the steps in the translation of RNA into protein. Capecchi and Gussin (1965) obtained support for Benzer and Champe's hypothesis that amber suppression was due to a mutation affecting an anticodon. They used a cell-free system with RNA from a suppressible mutant of the RNA virus *R*17 as messenger, and showed that no functional coat protein was synthesized unless serine *t*RNA from a suppressor strain of *E. coli* was present. The hypothesis was confirmed by Goodman, Abelson, Landy, Brenner and Smith (1968), who showed that the mutation giving rise to a particular amber suppressor in *E. coli* changes the anticodon of a tyrosine *t*RNA from GUA to CUA (**G** is a guanosine derivative). As indicated in Table 14.9, this change allows the transfer RNA to register with the triplet UAG in the messenger instead of the tyrosine codons UAU and UAC, and so to insert tyrosine in the polypeptide at the position corresponding to the amber triplet. There was no other change in the nucleotide sequence of the *t*RNA. As indicated in § 14.15, two forms of tyrosine *t*RNA were found to occur in *E. coli*, both having the same anticodon but differing in the nucleotides at positions *l*2 and *l*3 in Fig. 14.9. In order to isolate sufficient material of the amber suppressor *t*RNA, use was made of an *E. coli* virus called ϕ80, a defective strain of which carries the amber suppressor gene. By infecting the host with this virus, cells containing large numbers of copies of this gene and its product were obtained.

This technique also enabled this *t*RNA to be labelled isotopically for nucleotide-sequence determination.

Goodman *et al.* found that a particular ochre suppressor also caused the insertion of tyrosine, and may be due to a different base change at the same position as that which produced the amber suppressor. Other amber suppressors are thought to result from similar changes in serine and glutamine *t*RNAs, corresponding to reading the codon UAG as if it were UCG or CAG. This conclusion is based on the work of Weigert, Lanka and Garen (1965) and Kaplan, Stretton and Brenner (1965), who discovered that amber codons in the phosphatase gene of *E. coli*, and the gene for the protein of the head of virus *T*4, respectively, can be translated as serine, glutamine or tyrosine, according to which of three suppressor genes is responsible for suppression.

Duplicate genes for a particular *t*RNA seem to be necessary for the suppression of mutations causing premature polypeptide termination: the unmutated gene would allow translation of the normal codon for that *t*RNA. Goodman *et al.* found there were at least 3 genes for tyrosine *t*RNA, all normally giving rise to *t*RNAs with the same anticodon, although, as already mentioned, one had a slightly different nucleotide sequence elsewhere in the molecule.

The work of Goodman *et al.* not only establishes a mechanism for amber suppression, but provides direct experimental evidence for the position of the anticodon in *t*RNA, and shows, furthermore, that at least the first nucleotide of the anticodon is not necessary for recognition of the *t*RNA by its specific amino-acyl synthetase.

Hawthorne and Mortimer (1963) discovered some suppressor mutations in the yeast *Saccharomyces cerevisiae* which suppressed simultaneously certain mutants of several different genes. Magni and Puglisi (1967), from study of reverse-mutation, concluded that the genetic basis of this *super-suppression* in *S. cerevisiae* was probably modification of a transfer RNA so that it could translate a polypeptide-terminating codon and this has now been confirmed by Gilmore *et al.* (1968) from the amino-acid replacements resulting from super-suppression of a stop mutant* in the structural gene for cytochrome *c*.

The mechanism of releasing a polypeptide from the transfer RNA which brought the final amino-acid is not understood. Capecchi (1967) isolated a protein component required for the process. He used an amber mutant in the gene for the coat protein of the RNA virus *R*17. In a cell-free system, RNA from this mutant directs the synthesis of a peptide containing only 6 amino-acids, and by starvation of a chosen one of them, synthesis of the coat protein fragment can be stopped at any prescribed point. The substrate for the release factor was found to be the ribosome–messenger RNA–peptidyl-*t*RNA complex, implying that release occurred while the peptidyl-*t*RNA was still attached to the ribosome. A hypothetical polypeptide-terminating *t*RNA was not found. Capecchi raised the possibility that the stop codons (UAA, UAG and UGA) might be read, not by a transfer RNA, but by the release factor, or even by the ribosome itself. The further possibility was considered that these codons function because they cannot be read.

* Polypeptide-terminating mutants are often misleadingly called 'nonsense' mutants.

Bretscher (1968*a*) using an amber mutant which gave a hexapeptide fragment of the coat protein of RNA virus *f*2—a system similar to Capecchi's —showed that polypeptide termination is an active process and does not occur simply because a codon cannot be read. He purified the 6 transfer RNAs needed to synthesize the fragment, and used a cell-free protein-synthesizing system containing these 6 *t*RNAs and no others. Under these conditions the RNA of the amber mutant allowed the free hexapeptide to form, whereas with the RNA of the wild-type virus the hexapeptide remained attached to its transfer RNA. The codon in the wild-type gene at the position of the amber mutant was CAG for glutamine. This could not be read in the *in vitro* system owing to the absence of the glutamine *t*RNA, but this failure to read the CAG codon did not lead to release of the peptide from its transfer RNA. Evidently the UAG triplet of the amber codon is an active signal. Moreover, the method of purifying the 6 *t*RNAs would have eliminated a hypothetical polypeptide-terminating *t*RNA, and yet release of the hexapeptide occurred with the amber mutant. Bretscher concluded that there was no transfer RNA for polypeptide termination and that the stop codons were read in some other way.

§ 14.18 Conclusion

The theory of consecutive non-overlapping triplets of nucleotides as the amino-acid code is now established and all the 64 triplets have been allocated, 61 to amino-acids and 3 as stop signals in polypeptide synthesis. There are strong indications that the code deciphered for *Escherichia coli* is likely to be the same in all organisms. Much is known of the remarkable mechanism by which nucleotide sequence in DNA is first transcribed into messenger RNA and then translated into amino-acid sequence by means of transfer RNAs, acting in association with ribosomes. Notable gaps in knowledge are how far the mechanism of polypeptide initiation established for *E. coli* also applies to chromosomal organisms, what part of transfer RNA is recognized by the specific enzyme which catalyzes the attachment of the appropriate amino-acid, and how the stop signals operate. Much remains to be discovered about the structure, synthesis and functioning of ribosomes.

From what is already known of the mechanism for constructing a polypeptide from the information encoded in nucleic acid, it seems likely that several hundred different protein molecules are needed in order to synthesize even one such protein! These proteins, as well as being involved in large numbers in ribosome construction and activity, including the formation of the peptide bond itself, also catalyze transcription, the methylation and other changes in particular nucleotides in each transfer RNA, the synthesis of amino-acids, the attachment of each amino-acid to its transfer RNA, and the formylation of methionine after it has been joined to the polypeptide-initiating transfer RNA. How this interlocked mechanism evolved is a fascinating riddle.

15. *The theory of the operon*

§ 15.1 The bacterial operon

Demerec and associates (1955) obtained large numbers of nutritionally-deficient mutants of *Salmonella typhimurium*. Initially, ultraviolet light was used as mutagen, but subsequently it was found that the spontaneous mutation-rate was adequate, since mutants were readily selected from large populations of cells. The selection technique involved incubating the bacteria in minimal medium containing penicillin, which is lethal to growing cells but harmless to auxotrophic mutants, which were not metabolising because they were unable to grow on minimal medium.

When two mutants were grown in mixed culture, recombinants were often obtained. It was established that these were occurring as a result of transduction, that is, transfer of small fragments of the hereditary material from one cell to another by a virus (*P*22) (cf. § 16.3). By means of this recombination, detailed mapping of mutant sites was possible. It was found that there were numerous linearly-arranged sites of mutation within each functional unit (cistron). This was comparable to results with *Aspergillus nidulans* and the *T*-even viruses of *Escherichia coli* discussed in Chapter 13. However, an unexpected discovery made by Demerec and Demerec (1956) with tryptophan-requiring mutants, and by Hartman (1956) with histidine-requiring mutants, was that cistrons concerned with successive steps in the synthesis of these amino-acids were closely linked, and moreover were often arranged on the linkage map in the same order as the steps which they control in the biosynthetic pathway. This was in complete contrast to the results with chromosomal organisms, where, for example in *Neurospora*, the genes concerned with the various steps in the synthesis of specific amino-acids appeared to be scattered more or less at random through the linkage-groups.

Series of closely-linked genes arranged in the same order as the steps which they control in a biochemical sequence have since been found to be of frequent occurrence in bacteria, including the actinomycete *Streptomyces coelicolor* (Hopwood and Sermonti, 1962). Such linkage, however, is not found for all the steps in every pathway, and appears to be lacking in *Pseudomonas aeruginosa* (Fargie and Holloway, 1965).

Ames and Garry (1959) discovered that the amount of each of the enzymes concerned in histidine synthesis in *Salmonella* could vary over a wide range depending on the growth conditions, but the ratio of the activity of one enzyme to that of another was constant. They suggested that the cluster of

genes acted as a unit of regulation. The rate of production of the enzymes decreased (repression) as the amount of the final end-product, histidine, increased, and the integrated action of histidine on the group of enzymes has been called *co-ordinate repression.*

Jacob, Monod and associates (see Jacob and Monod 1961, 1962) found that mutants of *Escherichia coli* unable to make use of lactose were due to mutations in one or other of a series of 3 closely-linked genes, *z*, *y* and *a*, each concerned with the production of a different enzyme (β-galactosidase, a permease, and a transacetylase, respectively—see Fig. 15.1(*a*)). These enzymes are normally produced only in response to an inducer (lactose or other β-galactoside). In addition to mutants deficient in the ability to synthesise one of these enzymes, other mutants were found which formed all the lactose enzymes constitutively, that is, without the need of inducer. These mutants were found to be of two kinds.

One class of constitutive mutants was due to mutation at sites placed at one end of the series of lactose genes. Jacob *et al.* (1960) called this region an *operator* and the neighbouring group of genes which it affected in an integrated way they called an *operon.* Jacob and Monod (1961) found that the rate of

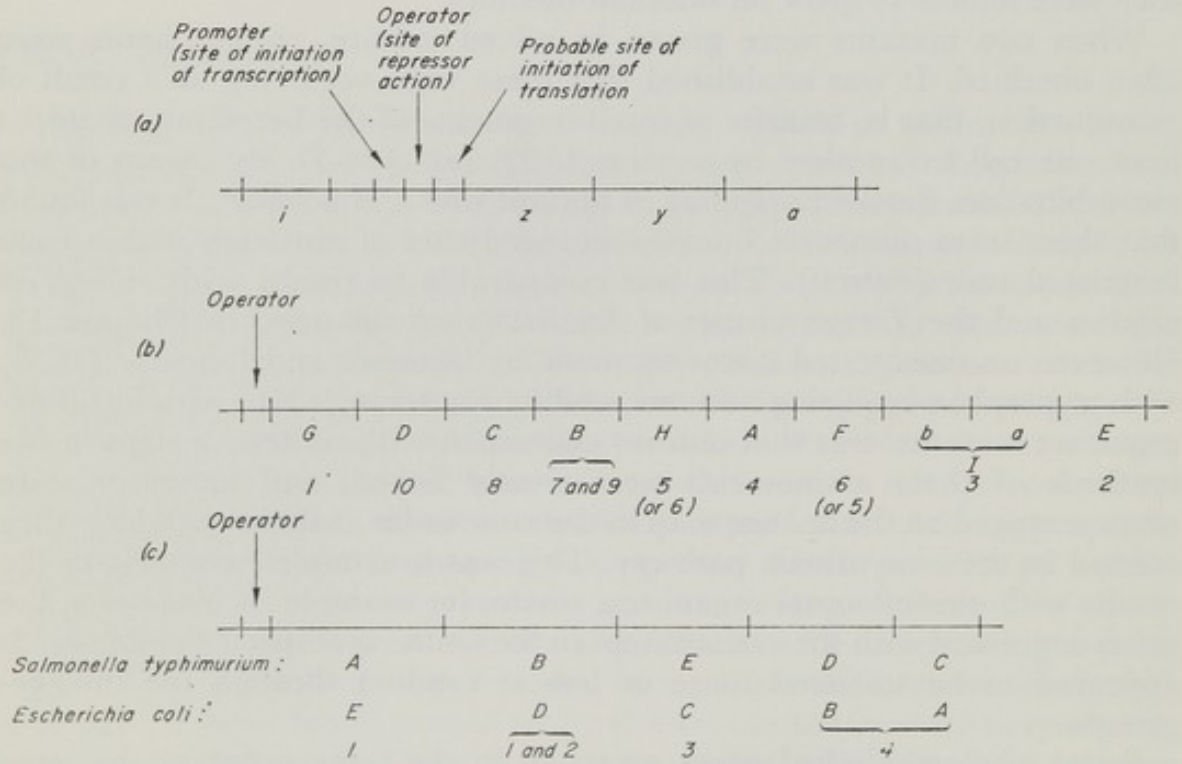


FIGURE 15.1 The diagrams show maps of some bacterial operons: (*a*) the lactose operon in *Escherichia coli* and its regulatory gene, *i*; (*b*) the histidine operon in *Salmonella typhimurium*; and (*c*) the tryptophan operons in *S. typhimurium* and *E. coli*. Genes are indicated by letters, and the sequence of action in the biosynthetic pathway of the enzymes for which they code is indicated by numerals. Diagram (*c*) is approximately to scale according to estimates by Imamoto and Yanofsky (1967) of the size of the *E. coli* tryptophan genes, based on the sub-unit molecular weights of their products; gene *A* is known to have 801 nucleotide-pairs, since it codes for a polypeptide containing 267 amino-acids (see § 13.8).

production of the three enzymes was much increased by lactose, but that the ratio of the activities of the different enzymes was constant. This *co-ordinate induction* was attributed to control by the operator. The co-ordinate repression, for example, of the histidine enzymes in *Salmonella* by histidine, was similarly explained. By study of heterozygotes for both an operator and an operon mutant in the *E. coli* lactose system, Jacob and Monod found that the operator directly influenced the genes of the neighbouring operon. Mutation in one particular cistron of this operon causes a failure of formation of the enzyme β-galactosidase, and as already mentioned, mutation in the operator causes the lactose enzymes to be formed constitutively (operator constitutive, o^c) instead of only in response to inducer. In cells heterozygous for these mutants (partial heterozygotes occur following conjugation—see § 16.3), the operator mutant was found to affect the lactose operon in its own chromoneme*, but not that in the homologue. Denoting the β-galactosidase-deficient mutant by z, they found that the cis configuration $\left(\frac{z\ o^c}{+\ +}\right)$ was normal, that is, β-galactosidase was formed only in response to inducer, but in the trans configuration $\left(\frac{+\ o^c}{z\ +}\right)$ β-galactosidase was formed constitutively.

The second class of constitutive mutants was found by Pardee, Jacob and Monod (1959) to be due to mutation at a gene locus called i (inducer) which was also closely linked to the lactose operon. Double heterozygotes for i and z mutants were found to be normal, that is, they formed β-galactosidase only in response to inducer, whether the mutants were in the cis $\left(\frac{z\ i}{+\ +}\right)$ or the trans $\left(\frac{+\ i}{z\ +}\right)$ configuration.

Another kind of mutant of the i gene, called i^s, or super-repressed, was found not to form the *lac* enzymes even in the presence of a β-galactoside.

§ 15.2 Jacob and Monod's theory

Jacob and Monod (1961) proposed that the i gene specified the structure of a repressor molecule which could bind to the operator of the lactose operon and thereby prevent the formation of the products of the z, y and a genes. It was suggested that the repression occurred by blocking the transcription of messenger RNA from the genes of the operon. It was further suggested that a β-galactoside (the inducer) binds to the repressor molecule so as to remove it from the operator, thereby allowing transcription and translation of the genes of the operon to occur.

According to this hypothesis the operator-constitutive (o^c) mutants have a defect in the operator such that the repressor can no longer bind to it. Conversely, the repressor-negative (i^-) mutants have a defect in the repressor which prevents it from binding to the operator. With either class of mutant, large amounts of the *lac* enzymes will be synthesized even in the absence of inducer, in agreement with observation. That the o^c mutants affect only

* Chromoneme = bacterial (or viral) chromosome—see § 11.1.

the immediately adjacent genes, while the i^- mutants act in the trans configuration as well, is an agreement with this hypothesis, because the *i* gene product (the repressor) could diffuse to the lactose operator on another chromoneme as readily as to that on its own, but diffusion does not apply to operator mutations. In i^s mutants, which never form the *lac* enzymes, the repressor was presumed to have lost its affinity for the inducer.

An essential feature of this hypothesis for the control of enzyme activity is that messenger RNA should be extremely unstable, such that enzyme synthesis stops within 2 or 3 minutes of stopping the synthesis of the messenger. The instability would presumably be on account of rapid breakdown by the enzyme ribonuclease (see § 15.4). For the lactose operon there is evidence from the work of Hayashi *et al.* (1963), Attardi *et al.* (1964) and others that the amount of messenger RNA present can vary, depending on the presence of inducer or other conditions. The messenger RNA was isolated by the technique described in § 14.6 of hybridization with homologons DNA.

Gilbert and Müller-Hill (1966) isolated the repressor of the lactose operon by an ingenious technique. An *i* gene mutant was obtained which had a greater than normal affinity for isopropylthiogalactoside (IPTG), a substance which is not used by the bacterium but will act as an inducer. An extract of the mutant was allowed to reach dialysis equilibrium with radioactive IPTG. More radioactivity was found in association with the extract within the dialysis sac than outside. This showed that a component of the extract could bind the inducer, and by using the binding as an assay, it was possible to purify the repressor. It was found to be a protein with a molecular weight of 150,000–200,000. In a control experiment, an extract of an i^s mutant did not concentrate IPTG. From the yield of repressor after the first steps of purification, Gilbert and Müller-Hill estimated there were about 10 repressor molecules per copy of the *i* gene in the cell, assuming there are two sites for the inducer on each repressor molecule.

Another repressor molecule has been isolated and also found to be a protein. This repressor controls the activity of virus λ of *E. coli*. This virus can exist in two states: integrated in the host chromoneme as the dormant provirus, or free in the cell (see § 16.3) where it can multiply rapidly and kill the cell. Jacob and Monod (1961) proposed that the inactivity when in the proviral state of all but one of the 40 or 50 genes in the virus is due to a repressor molecule determined by the one active gene called c_I. The messenger from this gene is indicated by the arrow labelled 1 in Fig. 14.6. The repressor molecules were believed to act at operators situated at the tails of the arrows labelled 2 in the figure*, and to do so not only in the provirus but also in any other λ virus that enters the cell, with the result that the cell is immune to destruction by the virus. Ptashne (1967*a*) eliminated most of the protein synthesis of host cells carrying λ provirus by ultraviolet irradiation to inactivate their DNA, and he then added a high concentration of λ, giving about 30 virus particles per cell, and the culture was supplied with radioactive (^{3}H) leucine. The irradiation followed by the heavy λ infection should mean that a high proportion of the label incorporated

* Ptashne and Hopkins (1968) confirmed the existence of these two operators, which are situated on either side of c_I and just to the right of gene *N* in Fig. 16.4.

into protein would be in the repressor molecules. It was necessary to use defective virus, unable to kill the host, because initially there are not enough repressor molecules to cope with 30 λ particles at once. Another similar culture was set up using in turn various $\lambda\, c_I$ amber mutants which prevent synthesis of the c_I product. This culture was supplied with ^{14}C-leucine. After an hour's growth, fractionation of the proteins from the two cultures on a diethylaminoethyl (DEAE) ion exchange column revealed similarity, except for a major peak of ^{3}H not shown by ^{14}C: this protein peak was therefore presumed to be the product of the c_I gene. Subsidiary experiments confirmed this. Temperature-sensitive c_I mutants which produce modified repressor *in vivo* gave a different chromatographic pattern for this protein peak on the DEAE column, thus identifying this protein as the c_I gene product and hence as the λ repressor. The repressor was found to be an acidic protein with an estimated molecular weight of 30,000.

Ptashne (1967*b*) mixed λ DNA with labelled λ repressor and from analysis on a sucrose gradient he found repressor bound to the DNA. With DNA of a λ-434 hybrid virus, which contains most of the λ genes but has virus 434's sites for accepting the repressor and so is sensitive to 434's repressor but not λ's, he found no such binding to λ repressor. He also showed that the λ repressor cannot bind to denatured λ DNA, that is, when its two chains have been separated. This work has thus established that the repressor binds specifically and with high affinity to λ DNA, in precise agreement with one of the primary features of the Jacob–Monod model, namely, that the repressor binds directly to the DNA of the operator.

Gilbert and Müller-Hill (1967) found that the *lac* repressor also binds directly to the operator DNA. The repressor was labelled with radioactive sulphur (^{35}S) by growing the bacteria in labelled medium, and then it was mixed with DNA containing the *lac* operator, and the mixture centrifuged in a glycerol gradient. The DNA had been obtained in quantity by using a defective strain of ϕ80-λ hybrid virus which contained the *lac* operon part of its host's DNA. The DNA, being larger, sedimented faster than protein, but carried some of the labelled protein bound to it, and this was evidently the *lac* repressor. Some further experiments confirmed this: first, addition of IPTG specifically released the protein from the DNA; second, DNA from ϕ80-λ carrying an operator-constitutive (o^c) mutant of the *lac* operon showed only a weak affinity for the protein; and third, virus DNA not carrying the *lac* operon showed no binding of protein.

Gilbert and Müller-Hill's remarkable experiments established not only that the product of the *i* gene is a protein, but moreover that it specifically binds to the DNA of the *lac* operator, in direct agreement with the Jacob–Monod theory. No binding was obtained when the DNA chains had been separated by denaturing. Gilbert and Müller-Hill concluded that the repressor protein binds to a specific sequence of duplex DNA. They argued that this sequence must be at least 11 or 12 base-pairs long to give the necessary specificity, allowing about 3×10^6 base-pairs in the *E. coli* chromoneme ($4^{11} = 4{\cdot}2 \times 10^6$ approx). Estimates of the binding constants led to the conclusion that the inducer must actively trigger the release of the repressor from the operator.

Gilbert and Müller-Hill's experiments with the *lac* repressor and Ptashne's with the λ repressor have greatly strengthened Jacob and Monod's theory. Many less simple hypotheses had been proposed, such as that the repressor blocks translation rather than transcription, by binding to the messenger RNA or to transfer RNAs, or by destroying the messenger.

With pathways which are co-ordinately repressed by the end-product, as in the histidine and tryptophan operons, Jacob and Monod proposed that the product of a regularoty gene cannot by itself bind to the operator but must first combine with the end-product of the pathway. Other more complex control mechanisms are discussed in § 15.7.

Jacob and Monod recognised two kinds of genes: *structural genes*, such as *z*, *y* and *a* in the lactose operon, which determine the amino-acid sequence of specific proteins, and *regulatory genes*, such as *i* and c_I which control the functioning of the structural genes. Gilbert, Müller-Hill and Ptashne have confirmed this distinction, and have shown that the *i* and c_I regulatory genes are the structural genes for the repressor proteins. Garen and Otsuji (1964) pointed out that a regulatory gene might not produce a repressor directly, but might be involved in a reaction required for repressor formation. They identified a regulatory gene of this kind affecting the synthesis of alkaline phosphatase (see § 15.7).

§ 15.3 Polar mutants

Franklin and Luria (1961) and Jacob and Monod (1962) found that some mutations in the β-galactosidase gene (*z*) of *Escherichia coli* in addition to preventing the synthesis of this enzyme, gave rise to reduced rates of synthesis of the other two lactose enzymes. Some permease (*y*) mutants had a reduced level of transacetylase, but β-galactosidase was unaffected. Thus, the mutants were polarised in their effects, only enzymes made by genes on the distal side of the mutant site with respect to the operator being reduced in amount. Beckwith (1964*b*) showed that a class of mutants which Jacob and Monod had called operator zero (o^0) mutations, because they prevent the formation of all the enzymes of the operon, appear to be polar mutants with the mutant site situated in the β-galactosidase gene near the operator, and with exceptionally strong effects in lowering the levels of activity of the *y* and *a* genes.

The histidine operon in *Salmonella typhimurium* has been intensively studied by Hartman, Ames and associates (see Loper, Grabnar, Stahl, Hartman and Hartman, 1964). Over 1000 histidine-requring mutants have been isolated. The operon has been found to consist of at least 10 genes specifying the enzymes of the pathway (Fig. 15.1(*b*)). There is at least one instance of a pair of genes determining one enzyme, and one gene specifies two enzymes, a dehydrase (no. 7) and a phosphatase (no. 9). It appears that different parts of the same protein catalyse different reactions (see § 13.7). Numerous polar mutants have been found, and the direction of their effect indicates that the operator is at the left-hand end of the operon in Fig. 15.1(*b*).

The trytophan operons in *S. typhimurium* and *E. coli* are illustrated in Fig. 15.1(*c*). They are alike as regards the sequence of genes coding for the enzymes of the pathway. Polar mutants place the operator at the left-hand end in the diagram in both species. Moreover, the *A* gene in *E. coli*, which codes for one of the two polypeptides of tryptophan synthetase, has been orientated with respect to the direction of messenger synthesis (see § 14.14) and this confirms that the operator is to the left.

Jacob and Monod (1961) favoured the idea that a single messenger RNA molecule was formed corresponding to an entire operon, because this would provide a neat explanation of the co-ordinate induction or repression of the enzymes of the operon. Attardi, Naono, Rouvière, Jacob and Gros (1964) isolated the messenger RNA corresponding to the lactose operon of *E. coli* by means of the technique developed by Hall and Spiegelman (1961) whereby messenger RNA forms hybrid molecules with homologous DNA when a heated mixture is slowly cooled (see § 14.6). Attardi and associates found the sedimentation coefficient of the messenger RNA was such as to suggest that it corresponded to the entire operon. Likewise, Martin (1964), using differential labelling with ^{14}C and ^{3}H to distinguish the RNA of a mutant deficient for the *S. typhimurium* histidine operon from that of a non-deficient strain, found by chromatography on a methyl albumin column, followed by centrifuging in a sucrose gradient, that the histidine operon messenger RNA had a sedimentation coefficient appropriate for the entire operon. Imamoto, Morikawa and Sato (1965) isolated the tryptophan messenger RNA of *E. coli* and found by density gradient centrifugation that it had a sedimentation coefficient of 33*S*, which again is appropriate for the whole operon. Mutants with deletions of parts of the operon had smaller messengers. By sedimentation in a sucrose gradient, Kiho and Rich (1965) measured the relative size of the ribosome aggregates (polyribosomes) showing β-galactosidase activity in *E. coli*. They found that strains with deletions in the *y* and *a* genes had smaller polyribosomes. They concluded that the *lac* messenger was multigenic in translation as well as transcription, that is to say, it did not break up into gene-length segments for polypeptide formation.

Beckwith (1964*a*) discovered that some of the polar mutants of the lactose operon of *E. coli* were suppressed by amber suppressors (§ 14.17) and were evidently polypeptide-terminating mutants. Extensive studies have since been made of the effects of such stop mutants in three operons: in the lactose operon by Newton, Beckwith, Zipser and Brenner (1965), in the tryptophan operon of *E. coli* by Yanofsky and Ito (1966) and in the histidine operon of *S. typhimurium* by Martin, Silbert, Smith and Whitfield (1966). All the results were similar and may be summarised as follows:

(*a*) The effects of amber (UAG) mutants were indistinguishable from those of ochre (UAA) mutants, and both invariably reduced the relative rates of synthesis of all proteins specified by genes situated on the distal side of the stop mutant with respect to the operator. As mentioned in § 14.17, Sambrook, Fan and Brenner (1967) found UGA mutants were also polar.

(*b*) For any particular stop mutant, all the genes on the non-operator side of the one containing the mutation had their activities lowered to the same extent.

(*c*) Suppressors of stop mutants suppressed the polar effect to the same degree and with the same specificity as they suppressed polypeptide termination.

(*d*) Within each gene there was a gradient of polarity, such that stop mutants situated near the operator end of a gene were more strongly polar (that is, reduced to a greater extent the rates of synthesis of the enzymes coded by the operator-distal genes) than those near the non-operator end, the gradient beginning afresh in each gene.

(*e*) The gene nearest the operator had a much steeper gradient of polarity than the others. This was first clearly demonstrated by Yanofsky and Ito (1966) with the *E. coli* tryptophan operon, and was shown for the histidine operon of *S. typhimurium* by Fink and Martin (1967).

Yanofsky and Ito discovered that two stop mutations present simultaneously in different genes of the operon showed polar effects independently, the percentage reduction in the relative rates of activity of distal genes caused by the second mutation being superimposed on the reduction resulting from the first mutation. On the other hand, two such mutations in the same gene behaved as if only one were present.

Zipser and Newton (1967) showed in an ingenious way that the gradient of polarity within a gene is dependent on the distance of the stop mutant from the distal end of the gene and not on the distance from the operator end. They found that the polarity caused by stop mutants in the *z* gene of the lactose operon was unaffected by deletions of segments of *z* on the operator side of the stop mutant, but was greatly reduced by deletions situated within *z* on the distal side. Such deletions reduced the distance to the non-operator end of the gene.

Ito and Crawford (1965), from study of the tryptophan operon of *E. coli*, discovered that polar mutants can also cause a short range antipolar effect, that is, the enzyme specified by the gene adjacent to the mutated gene on the operator side was formed in reduced amounts. Yanofsky and Ito (1967) found that stop mutants in gene *A* (see Fig. 15.1(*c*)) affected gene *B* and to a lesser extent gene *C*, but that *D* and *E* were unaffected. This falling off in antipolarity with distance is in contrast to polarity (see item (*b*) above). On the other hand, antipolarity showed the same dependence as polarity on the position of the stop mutant within the gene, being greatest when the mutant was far from the distal end. Antipolarity, like polarity, was also partially relieved by specific suppressors of the stop mutant. Fink and Martin (1967) did not find antipolarity in the histidine operon of *S. typhimurium*.

Ito and Crawford also discovered there was no polar effect with mutations in the tryptophan operon of *E. coli* that gave rise to cross-reacting material, that is, protein that could be recognised immunologically as similar to the normal product of the gene. Conversely, polar mutants produced no cross-reacting material. Likewise, Martin *et al.* (1966) found no polarity with mutations in the *C* gene of the histidine operon of *S. typhimurium* that gave cross-reacting material. Such mutations are thought to be base substitutions

causing an amino-acid substitution at one point in the polypeptide. Unlike stop mutants, these 'mis-sense' mutants do not respond to amber or ochre suppressors and are often leaky, that is, allow some growth to occur.

Martin *et al.* (1966) found that another class of mutants, in addition to stop mutants, were polar. These were phase-shift mutants (§ 14.3 and § 14.13). They were recognised by their failure to revert with base-substituting mutagens, their lack of suppression through mutation of another gene (such as gives rise to amber and ochre suppressors), and by their lack of cross-reacting material. Martin *et al.* suggested that the polarity observed with phase-shift mutants was due to a stop codon created by the change of reading-frame. Martin (1967) obtained support for this idea from study of revertants of phase-shift mutants. Such revertants will be expected to have an addition of 1 or 2 nucleotides in proximity to a corresponding deletion (cf. Fig. 14.14) and not to be polar: in order to be polar the plus and minus phase-shifts would need to straddle the postulated stop codon generated by the first phase-shift, but this would not produce a revertant because the second phase-shift would not be read. The revertants were indeed non-polar.

It thus seems likely that polar effects in operons may all be due to stop codons* arising within a gene, either directly through an appropriate base change, or indirectly through a phase-shift following upon the addition or deletion of a number of nucleotides other than 3 or a multiple of 3.

The discovery that stop mutants and phase-shift mutants show a reduced level of activity of operator-distal genes but not a complete absence of these gene products (except when the stop mutant is at the operator end of the first gene) implies that there must be polypeptide-initiating regions at the beginning of each gene of an operon. The existence of re-initiating regions within a messenger has been demonstrated: as mentioned in § 14.16, Adams and Capecchi (1966) showed that formylmethionine was involved in the initiation of the polypeptides of the multigenic messenger of RNA virus *R*17 of *E. coli*, and Sarabhai and Brenner (1967) isolated an *r*II *B* mutant of virus *T*4 that permitted initiation of a second polypeptide after a stop mutant in the *B* gene.

§ 15.4 The hypothesis of transcription coupled to translation

Attardi *et al.* (1964) found that two strongly polar mutants situated at the operator end of the *z* gene of the lactose operon of *E. coli* apparently contained no *lac*-specific messenger RNA after induction. This finding conflicted with Beckwith's (1964*a*) discovery that these mutants could be suppressed by stop-mutant suppressors and therefore were blocked in translation rather than in transcription. Beckwith suggested that the messenger RNA transcribed from the lactose region was particularly unstable in these mutants and so escaped detection. The gradient of polarity within *z* found by Newton *et al.* (1965) would then be accounted for if messenger breakdown depended on the distance between the site of the mutation and the start of the next gene, perhaps because ribosomes normally protected the messenger from ribonuclease attack.

* An exception to this generalization is known: Saedler and Starlinger (1967) found polar mutants of another kind in the galactose operon of *E. coli*.

Another possibility, proposed by Ames and Hartman (1964), was that ribosome movement along the messenger RNA might be necessary to dissociate the messenger from the DNA after the RNA had been synthesized by RNA polymerase. Failure of translation might therefore hinder or even block messenger synthesis. This idea that transcription may be coupled to translation has gained in plausibility with the discovery (§ 14.9 and § 14.14) that these processes occur in the same direction. To explain the gradient of polarity within *z*, Newton *et al.* (1965) suggested that failure to release the messenger from the DNA might depend on the distance to the next start signal, that is, the beginning of the next gene.

According to these transcription models of polarity, which Newton *et al.* (1965) favoured, the messenger is able to pick up ribosomes at the start of each gene.

Imamoto and Yanofsky (1967) found from DNA–RNA hybridization that polar mutants of the tryptophan operon of *E. coli* were deficient in the messenger corresponding to the genes on the operator-distal side of the mutated gene, and moreover, the proportion of such short messengers was greater with the more strongly polar mutants. Pulse-labelling experiments gave results which suggested that breakdown of messenger was not occurring, and so Imamoto and Yanofsky favoured the coupling of transcription to translation as an explanation of polarity. Baker and Yanofsky (1968) showed, however, that degradation of the *tryp* messenger, which can begin within 30 seconds of the completion of its synthesis, occurs sequentially from the 3′ end, that is, the operator-distal end. They therefore considered that polar mutants might lead to rapid degradation of the distal part of the messenger. Nevertheless, a purely translational model of polarity, called the modulation hypothesis, also has much support.

§ 15.5 The modulation hypothesis

Ames and Hartman (1964) suggested that, at or near the junctions between genes in the messenger RNA from an operon, there may be a special triplet of nucleotides which they called a modulating triplet, and that this may cause the reading of the message to slow down or stop at this point. They suggested that ribosomes can associate with the messenger only at the operator end, and so, assuming the reading of the message begins at that end, the effect of the modulating triplets will be that genes near the operator will give rise to relatively large numbers of enzyme molecules and genes at the other end of the operon to the same number or fewer, but never more. They argued that this mechanism of control enabled a cell to synthesize unequal numbers of the enzyme molecules of a pathway, and yet control their production co-ordinately. The sequence of genes in an operon would correspond to that of the efficiency of their products: the least efficient, requiring most molecules, would be expected to be coded by the gene nearest the operator, as a result of selection favouring the optimal gene sequence. According to Martin *et al.* (1966) no exceptions have been found to the prediction that distal

genes will not give rise to more protein molecules than genes situated nearer to the operator in the same operon.

Ames and Hartman suggested that polar mutants were due to mutation of a normal triplet to give a modulating triplet.

Martin *et al.* (1966) proposed that the modulating triplets were stop codons and that the slowing down of the reading of the message, which these triplets were supposed to cause, was due to ribosomes leaving the messenger in the untranslated region between a stop codon and the start of the next gene. The probability of ribosome detachment was thought to depend on the length of the untranslated region. Yanofsky and Ito (1966) independently proposed the same modifications to the modulation hypothesis. Martin *et al.* believed that polypeptide-initiating regions may be of several types and that their efficiency as initiators affects the magnitude of the polar effect of stop mutants in the preceding gene.

The modulation hypothesis fails to account for the shorter messenger often found with stop mutants, and it does not explain why the first gene in an operon shows a steeper polarity gradient than the others. Yanofsky and Ito (1966) therefore suggested that ribosome travel past the initial segment of the messenger, that is, of the gene nearest the operator, was required to initiate release of the messenger from DNA.

Fink and Martin (1967) also favoured a composite model incorporating both the coupling of transcription to translation and modulation in translation. They regarded the coupling model by itself as unsatisfactory because it did not explain the steep polarity gradient in the first gene compared with other genes even when, as they had found with the histidine operon in *S. typhimurium*, the first gene (*G*) may be of the same length as a more distal one (*A*). The hypothesis of transcription coupled to translation predicts a rapid fall-off in polarity as stop mutants closer to the distal end of the gene are considered, in agreement with observations for the first gene in the *E. coli* lactose and tryptophan operons and the *S. typhimurium* histidine operon, but not in agreement with the polarity gradient in other genes. In order to explain the shallow gradient of polarity in internal genes of an operon, Fink and Martin suggested that once transcription had reached some critical point, it became uncoupled from translation. Polarity in the first gene of an operon was explained primarily by transcription being coupled to the blocked translation, whereas in other genes it was attributed to translation modulation. They suggested that the critical distance along the operon where transcription is uncoupled from translation might be the distance which allows the synthesis of a second messenger to be initiated at the operator. The formation of the second messenger might substitute for ribosome travel as a means of dissociating the first messenger from the DNA.

The discovery that the RNA of RNA viruses such as *f*2 and *R*17 of *E. coli* is analogous to a multigenic message of their host, and that this RNA codes for 3 viral proteins, has allowed polarity to be studied *in vitro* (see Engelhardt, Webster and Zinder, 1967 and Lodish, 1968). These studies point rather strongly to a translational model of polarity such as the modulation hypothesis, and promise to elucidate one of the key questions: whether

ribosomes can become associated with the messenger at intermediate points or only at the 5′ end.

§ 15.6 The promoter

Jacob, Ullman and Monod (1964) discovered that when a deletion in the lactose operon of *E. coli* covers the operator and the neighbouring end of the *z* gene, activity of the remaining genes (*y* and *a*) is possible only if the deletion extends beyond *i* (Fig. 15.1(*a*)) into another operon such as the purine operon or the tryptophan operon. If *y* and *a*, in the absence of the operator and hence not repressed, could function by connecting them to any region of DNA, one would expect to find deletions starting in *z* which ended either in the operator or in whatever length of DNA may exist between the operator and *i*, but no such deletions were found. Jacob *et al.* concluded that there was a site in the neighbourhood of the operator which was necessary to allow the *lac* genes to be expressed. Removal of this site inactivates the operon, unless the corresponding site of another operon is substituted by means of a long deletion.

Jacob *et al.* also found that o^c mutations, which destroy the function of the operator so that the lactose genes function without the need of inducer, did not reduce the maximal rate of operon expression. This implied that the operator, as the site of repressor action, was not the site which determines this rate. The proposed the term *promoter* for the site for the initiation of operon expression. The term was subsequently defined more precisely (see Ippen *et al.* 1968) as the site for initiating the synthesis of messenger RNA.

Ippen, Miller, Scaife and Beckwith (1968) isolated mutants which co-ordinately reduced the rate of expression of the *lac* genes. Like o^c mutants (§ 15.1), these mutants were *cis-dominant*, reducing the rate of expression of the operon on the same chromoneme but not on another one. It was concluded that the mutants had an alteration in a site essential for the initiation of either transcription or translation of the operon. The mutants were mapped by the use of a series of deletions of various extent and, contrary to previous ideas, found to lie between the regulator gene (*i*) and the operator (Fig. 15.1(*a*)). This position implies that they were promoter mutants, that is, defective in transcription rather than in translation, because it seems unlikely that the operator is translated into protein. Mutations of the operator (o^c) are not suppressed by amber or ochre suppressors, which are known to act at the level of translation by reading the stop codon as an amino-acid (§ 14.17). If the operator were translated, some amber or ochre o^c mutants would be expected, but none has been found. The position found for the promoter implies that transcription begins before the operator, so it is evidently transcribed. Ippen *et al.* suggested that the promoter might act as a binding site for RNA polymerase, the promoter mutants reducing the site's affinity for the enzyme. The repressor, in binding to the operator, could then block the movement of the RNA polymerase into the structural genes of the operon. Assuming that the operator is not translated, a point on the messenger between the operator and the first structural gene, *z*, would be the site for the initiation of translation.

Bauerle and Margolin (1967) discovered that in the tryptophan operon of *S. typhimurium* there is a secondary initiator of gene expression between genes *B* and *E* (Fig. 15.1(*c*)). Their main findings were as follows. The first two genes of the operon (*A* and *B*) form one co-ordinate unit of expression and the other three (*E*, *D* and *C*) another, which is not, however, wholly independent of *A* and *B*. Polar mutations in *A* depress the activity of *B* more severely than that of *E*, *D* and *C*. Deletions extending into *A* from the operator end have no *B* activity, but retain a reduced level of expression of *E*, *D* and *C* without control, however, by tryptophan. All the genes are inactive when deletions extend from the operator end beyond the *B-E* boundary. Deletions of the *B-E* junction render the remaining parts of the operon co-ordinate. Bauerle and Margolin concluded that there were two sites for initiation of gene expression, one at the operator end and the other between genes *B* and *E*. If these sites were promoters, that is, initiated transcription, the first would give a single messenger for all five genes, and the second an *E-D-C* messenger. On the other hand, if the sites initiated translation, the attachment of ribosomes to the messenger would be possible at either of them, but the source of the messenger when *E*, *D* and *C* functioned alone would be problematical. Thus, the transcription hypothesis seems the more plausible, the operon having two promoters, one associated with the operator and the other in mid-operon.

§ 15.7 Other bacterial systems for the control of gene activity

The negative control system by repression, now known to occur in *E. coli* with the lactose operon and virus λ, is not the only mechanism of control of gene action in bacteria.

Englesberg, Irr, Power and Lee (1965) studied the genetic basis of the use of L-arabinose by *E. coli* and found that the system differs in important respects from the lactose control mechanism. Genes for a kinase (*B*), an isomerase (*A*) and an epimerase (*D*) are closely linked in the order given and believed to form an operon. The enzymes function in the breakdown of arabinose in the sequence *A*, *B*, *D*. A regulatory gene (*C*), also closely-linked in the order *CBAD*, is believed to produce a repressor which attaches to the *ara* operator, from which it is removed by arabinose. Here the resemblance to the lactose system ends, because the repressor–arabinose complex is thought to have a positive function as an *activator*, promoting synthesis of the arabinose enzymes, which cannot occur merely in the absence of repressor. Furthermore, this activator promotes co-ordinate synthesis not only of the products of the three structural genes of the *ara* operon but also of a permease determined by a gene (*E*) situated in a different part of the linkage map. Englesberg *et al.* suggested that positive (activator) control is required for the co-ordinate control of genes in different operons. They assumed that gene *E* is part of another operon, and that the activator is needed to remove it from control by this other system. Their model is tentative, but the data clearly indicate a different organisation from that of the lactose genes.

The control of alkaline phosphatase synthesis in *E. coli* shows a different system again. Garen and Otsuji (1964) found there were three regulatory genes. One of these (*R*1) is closely-linked to the phosphatase structural gene and, from earlier studies, is thought to control the formation of an inducer required for the synthesis of the phosphatase. The other two (*R2a* and *R2b*) are closely-linked to one another in a different part of the chromoneme and are thought to control the formation of an enzyme that can convert the inducer into a repressor when a high concentration of orthophosphate is present. The product of gene *R2a* was shown to be a protein. Its formation was found to be repressed under the same conditions as the phosphatase, and so Garen and Otsuji considered that both were regulated by the same repressor. They suggested that this might explain how regulatory genes were themselves regulated.

Harris and Sabath (1964), from study of the synthesis of the enzyme penicillinase in *Bacillus cereus* in response to inducer (cephalosporin *C*), found that the antibiotic actinomycin *D* inhibited RNA synthesis within 1 or 2 minutes of its addition to the culture, but that synthesis of the enzyme continued for the cell generation-time (about 20 minutes). They concluded that the messenger RNA for penicillinase was stable and that in consequence the regulation of the synthesis of the enzyme could not occur by controlling messenger formation.

These results suggest that there are several different ways in which enzyme synthesis may be controlled and that, although the primary control is at transcription, a secondary mechanism may also exist acting at the level of the translation of messenger RNA into polypeptide.

§ 15.8 Viral operons

The existence of operons, defined by the occurrence of multigenic messenger, has been established in bacterial viruses. This is evident from the studies of the RNA viruses *R*17 and *f*2 of *Escherichia coli* referred to in § 15.4. The three proteins coded by the RNA of these viruses are diverse in function —they include the protein of the viral coat and an RNA polymerase—and there is no question of the operon representing a functional unit. On the other hand, in the DNA virus *T*4 several pairs of closely-linked genes are known in which amber mutants in one lower the activity of the other, and these genes are concerned with the same function: Stahl, Murray, Nakata and Craseman (1966) found that amber mutants in gene 51 depress the activity of gene 27 (see Fig. 18.3), both of which are concerned with the formation of the base-plate of the tail of the virus (King, 1968). Likewise, Stahl *et al.* found that amber mutants in gene 34 depressed the activity of gene 35. According to Edgar and Lielausis (1968) these genes affect the last two steps, 35's product before 34, in the formation of the tail-fibres. Similarly, Edgar and Lielausis found that amber mutants in gene 13 lower the activity of gene 14, both of which concern the final steps in the formation of the head of the virus.

In the viruses λ and *T*4, genes concerned with related functions are

frequently grouped together (see Figs. 14.6 and 18.3). The comparative rarity, however, with which amber mutants in one gene affect another suggests that these groups of genes usually do not have multigenic messengers, and so should not be regarded as operons. Stahl and Murray (1966), from complementation studies between mutants in viruses *T*2 and *T*4, argued that in the evolutionary divergence of these two closely-related viruses selection had favoured the maintenance of particular combinations of mutations in different genes, and so had favoured close linkage of these genes.

§ 15.9 The replicon

As a derivative of the idea of interactions between the products of regulatory genes and operators in the control of gene action, Jacob and Brenner (1963) made comparable postulates for the control of DNA replication. They suggested that replication is controlled in such a way that it occurs independently in each unit or *replicon*. Bacterial and viral chromonemes appear to be single replicons, but a chromosome contains a number of them (see § 12.7). Jacob, Brenner and Cuzin (1964), in elaborating the replicon concept, pointed out that the enzymes which make DNA and RNA copies from a DNA primer function in an uncontrolled way *in vitro*, but that *in vivo* both replication and transcription appear to be controlled with such precision and specificity that they cannot be regulated by the flow of their precursors. Jacob *et al.* postulated that DNA replication required, first, a structural gene controlling the synthesis of a specific *initiator*, which might be a specific DNA polymerase or a priming enzyme able to separate the chains of the double helix, and secondly, an operator of replication, or *replicator*, which would be a recognition site upon which the initiator would act, allowing the replication of the DNA attached to the replicator. This model for control of replication is based on the repressor model for the control of gene action, but positive regulation of replication is postulated instead of the negative regulation, by removal of a repressor, proposed for the lactose enzymes. The initiator and replicator were considered to be specific for each replicon, so that, for instance, the initiator for a bacterial virus would not recognise the replicator for its host. This hypothesis would account for the observation that chromonemal fragments, introduced into a bacterial cell by conjugation, transformation, or transduction, fail to replicate unless incorporated into the chromoneme of the acceptor cell (see § 16.3), since the fragments would be unlikely to include the replicator.

§ 15.10 The possible occurrence of operons in chromosomal organisms

The operon concept was developed by Jacob, Monod and associates to explain the integrated control of the action of genes in a biosynthetic pathway in bacteria. In chromosomal organisms, the genes concerned with successive steps in reaction chains appear, in general, to be scattered through the

linkage groups. This is well-established for *Neurospora crassa* and *Aspergillus nidulans*, and is indicated, for instance, by the frequency of 9:3:3:1 ratios (or modifications such as 9:7) in the F_2 generation in studies on the inheritance of anthocyanin pigmentation in flowering plants. Recently, however, some examples of operon-like organization have been discovered in fungi.

Ahmed, Case and Giles (1964) suggested that the histidine-3 region in the right arm of linkage group I of *N. crassa* was an operon containing 4 genes *A–D*, arranged in alphabetical sequence with *A* nearest the centromere, and concerned with steps 2, 3 and 10 in the histidine biosynthetic pathway. The other steps in the pathway are catalysed by the products of genes at scattered positions in 4 of the 7 linkage groups. The evidence for *his*-3 being an operon was as follows. Many of the mutations mapping in the segments *A*, *B* and *D* were defective in the enzymes catalysing steps 3, 2 and 10, respectively, and the mutants in any one of these classes complemented those in the other two. Other *his*-3 mutants were defective for more than one enzyme and some of these failed to complement with any *his*-3 mutants. These mostly mapped in segment *A* and were interpreted as polar mutants. Ahmed *et al.* concluded that each *his*-3 segment corresponded to a different polypeptide and that a single messenger RNA molecule was formed corresponding to *his*-3 as a whole, the promoter being at the left-hand (centromere-proximal) end next to gene *A*. Segment *C* was inferred to exist, and to code for a polypeptide required for both steps 2 and 3, from biochemical studies which indicated that certain mutants were defective in both these enzymes. Catcheside (1965), however, on the basis of his studies of *his*-3 mutants, pointed out that many different complementation maps could be drawn from the data and that it was not justifiable to infer polarity from their pattern. Moreover, he found that mutants defective for all the *his*-3 activities mapped in various positions and those defective for one function overlapped in position those defective for another. He favoured the explanation that *his*-3 determines the production of only one polypeptide having several enzymic functions.

Fink (1966) found that the *hi*-4 region in *Saccharomyces cerevisiae* resembled *his*-3 in *N. crassa*. Three segments *A*, *B* and *C*, mapping in that order, apparently coded for the enzymes catalysing steps 3, 2 and 10, respectively, in the histidine pathway. Polarity, with the promoter to the left, was inferred because (*a*) non-complementing mutants lacking all three enzyme activities mapped only in segment *A*, and (*b*) mutants defective in steps 2 and 10 were found, but not in steps 2 and 3. Only those mutants with polarized defects were suppressible by a probable stop-mutant suppressor, in agreement with the operon interpretation.

Giles, Case, Partridge and Ahmed (1967) studied mutants of *N. crassa* having a requirement for aromatic amino-acids. There are thought to be 7 steps in the *arom* pathway, each catalysed by a different enzyme: of these, steps 2–6 appeared to be controlled by a cluster of 5 genes in the right arm of linkage group II (Fig. 15.2). Giles *et al.* considered this *arom* region to code for 5 different polypeptides transcribed by one multigenic messenger from a promoter at the right-hand (centromere-distal) end. Their evidence for this conclusion was as follows. Mutants of the gene cluster were found

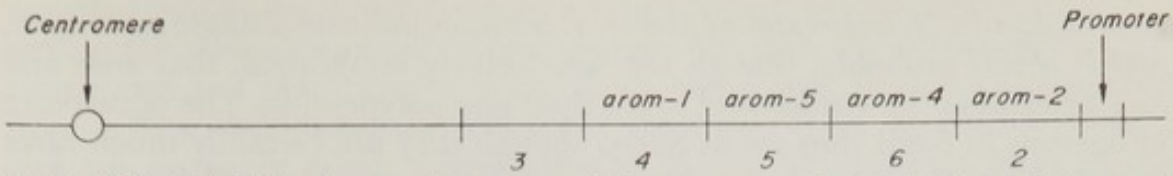


FIGURE 15.2 The diagram is a map of the *arom* gene cluster in the right arm of linkage group II of *Neurospora crassa* from the work of Giles, Case, Partridge and Ahmed (1967). The names of the genes are given above the line, and the sequence in which their products act in the biosynthetic pathway leading to the aromatic amino-acids is indicated by the numerals below the line. Mutants of the postulated gene for step 3 (dehydroquinase) have not been found (see text).*

either to be deficient for one enzyme, in which case they mapped in the appropriate segment of the cluster, or to be deficient for all 5 enzymes. These pleiotropic mutants were of various kinds. Some showed no complementation with any of the single-deficiency mutants, and these all mapped in the *arom*-2 gene at the right-hand end of the cluster; they were interpreted as polar mutants of this gene. Other pleiotropic mutants mapped in internal genes of the cluster and, in general, showed complementation only with single-deficiency mutants mapping to their right; they were considered to be polar mutants of the gene in which they mapped. In further support of this interpretation some of the pleiotropic mutants, but not the single-deficiency mutants, could be suppressed by a probable suppressor of stop mutants.

Density-gradient centrifugation revealed that the enzymes coded by the *arom* gene cluster form an aggregate with a molecular weight of about 200,000. The polar mutants, unlike the single-defect mutants, gave much smaller molecular weights: evidently stop mutants prevent the formation of the normal enzyme aggregate and so block all 5 steps, even though, as shown by the complementation data, normal enzyme molecules are usually formed by genes situated to the right of that containing the polar mutation.

No mutants were found which were deficient only for the step 3 enzyme.* This step is the conversion of 5-dehydroquinic acid to 5-dehydroshikimic acid by dehydroquinase. All mutants with reduced levels of this enzyme had marked reductions of the other four as well. From biochemical studies Giles, Partridge, Ahmed and Case (1967) concluded that *N. crassa* has two dehydroquinases—a constitutive enzyme in the synthetic pathway coded by a gene in the *arom* cluster, and an inducible enzyme in a degradative pathway coded by a different gene not in the *arom* cluster. No method has yet been devised, however, to detect mutants in either of these postulated genes.* The two enzymes were separated by sucrose gradient density centrifugation and found to differ in thermolability.

Giles, Case *et al.* pointed out that the presence of an enzyme aggregate distinguishes the *arom* gene cluster from most bacterial operons. Moreover, there is no evidence for regulatory genes or an operator in connection with the *arom* cluster. An operon, however, is probably best defined in terms of one messenger for more than one polypeptide. The polar mutants of the

* Rines (1968) has now obtained *arom-9* mutants (mapping in the *arom* cluster) which lack the synthetic dehydroquinase, and he has also obtained another class of mutants (not yet mapped) which lack the inducible dehydroquinase.

arom cluster in *N. crassa* and of *hi*-4 in *S. cerevisiae* indicate a single messenger, and it seems probable, though not conclusively established, that *arom* and *hi*-4 determine the synthesis of more than one polypeptide. The occurrence of operons in fungi thus seems likely, though they are certainly much rarer than in bacteria. Giles *et al.* suggested that an operon is an efficient method of coding for an enzyme aggregate, and that the *arom* enzyme aggregate in *N. crassa* provides a channelling mechanism for separating two potentially competing pathways—one synthetic and one degradative—which have one step in common. The occurrence of the *arom* operon may therefore be related to the existence of these two pathways.

In support of the idea that the *arom* operon in *N. crassa* has evolved in conjunction with the enzyme aggregate, Gollub, Zalkin and Sprinson (1967) found that the genes for the *arom* pathway were not closely linked in *Salmonella typhimurium*, and Giles, Case *et al.* found that the enzymes of the pathway did not form an aggregate either in *S. typhimurium* or *Escherichia coli*. Enzyme aggregates, however, are not necessarily associated with operons. DeMoss, Jackson and Chalmers (1967) showed that mutations in either of the genes *tryp-1* and *tryp-2* of *N. crassa*, which are in linkage groups III and VI respectively, can reduce the size and activity of an enzyme aggregate concerned with steps 1–3 of the tryptophan pathway. The *tryp-2* gene codes for anthranilate synthetase, which catalyses the first step, but interaction with the *tryp-1* gene product is required for expression of this enzyme activity. On the other hand, such interaction is not required for expression of the two enzyme activities (steps 2 and 3) of the *tryp-1* product.

An example of genes of related function showing linkage but not forming an operon is provided by the work of Pateman and Cove (1967) on nitrate reduction in *Aspergillus nidulans*. Nitrate is used by this fungus as a source of nitrogen, following reduction first to nitrite and then via hydroxylamine to ammonium ions. On the basis of their results, Pateman and Cove suggested that a regulatory gene produces a repressor which prevents the synthesis of nitrate reductase and nitrite reductase from their respective structural genes. Nitrate is necessary to convert the repressor into an inducer which allows synthesis of these enzymes. The combination of positive and negative control is similar to that proposed by Englesberg *et al.* (1965) for the arabinose enzymes in *E. coli* (see § 15.7). Unlike that system, however, the regulatory gene for the nitrate pathway in *A. nidulans* is only loosely linked to the structural genes, which also show 10% recombination with one another. Clearly there is no operon, each structural gene evidently having its own operator.

Some authors have suggested that the human β and δ haemoglobin polypeptides are the products of an operon. In the normal adult, 97·5% of the haemoglobin contains two α chains and two β chains and 2·5% two α and two δ chains (Ingram and Stretton, 1961). Mutant forms of the α chain show no linkage in inheritance with mutant forms of β (see § 13.7), but studies of the inheritance of mutant forms of β and δ, when these occurred in the same family, showed that the β and δ genes are closely linked. The β and δ polypeptides differ in only 10 of their 146 amino-acids: at positions 9, 12, 22, 50, 86, 87, 116, 117, 124 or 125, and 126. Moreover, Baglioni (1962) showed that in haemoglobin Lepore recombination had

occurred within the β and δ genes: a single hybrid polypeptide was formed having the normal number of amino-acids but with the δ sequence at the amino end and the β sequence at the carboxyl end. Lepore haemoglobin from a New Guinea family had less δ than that from an Italian subject, and has probably arisen independently. In the New Guinea form of the disease the recombination has occurred in the gene segments corresponding to the region between amino-acids 22 and 50, and in the Italian form between 87 and 116 (see Labie, Schroeder and Huisman, 1966). The occurrence of these recombinant forms of β and δ, with δ at the amino end of the polypeptide and hence at the 5′ end of the gene (expressed in terms of the messenger RNA) suggests that β and δ may be contiguous genes orientated the same way and with δ on the 5′ side of β. A condition called hereditary persistence of foetal haemoglobin shows depression of both β- and δ-chain production: in the form of this condition which occurs in Greece both β and δ production are moderately reduced, while in its African form both β and δ polypeptides are completely absent. The mutations responsible show close linkage to the β and δ structural genes. This has led to the proposal that δ and β might form an operon.

Other hereditary conditions, however, give contrary evidence. In the disease called thalassaemia the synthesis of either the α or the β polypeptide is lowered or suppressed. The disease is usually lethal when homozygous, but nevertheless is of widespread occurrence in the Mediterranean countries and the tropics, apparently because like sickle-cell anaemia (§ 2.6) the heterozygote has greater than normal resistance to malaria. In β-thalassaemia, besides the reduction in synthesis of β-chains, there is an increased synthesis of δ chains, both in cis and trans. This argues strongly against an operon for the β and δ genes. The mutation responsible for β-thalassaemia shows close linkage to the β and δ genes, although recombination has been detected with δ (see review by Motulsky, 1965). One possibility is that β-thalassaemia is due to a mutation in the promoter of the β structural gene, thereby reducing its affinity for RNA polymerase*. Any hypothesis of the nature of the genetic alterations in thalassaemia has to explain, however, the curious fact that haemoglobin Lepore is associated with the clinical and haematological features of thalassaemia.

There are two other human haemoglobin polypeptides: γ formed in foetal life and ε in embryonic life. Each is thought to be determined by a different gene, but it is not known whether the γ and ε genes show linkage to any of the other haemoglobin genes. Like β and δ, the γ and ε chains normally function in pairs associated with a pair of α chains. Baglioni and Campana (1967) believe that β chains are not released from polyribosomes until an α chain has combined with the β. This would provide a mechanism for co-ordinating α- and β-chain production. The mechanism of compensation, however, between β, γ and δ, by which reduced output of one of these polypeptides is often associated with increased output of another, is not understood.

Although operons seem to be of rare occurrence in chromosomal organisms

* Support for this hypothesis has been obtained by Clegg *et al.* (1968) who found by pulse-labelling that the rate of β-chain synthesis is normal in β-thalassaemics.

and the genes concerned with each biochemical pathway are usually widely scattered through the chromosomes, nevertheless, functionally related genes are not distributed completely at random. Elston and Glassman (1967) considered whether genes which control morphological structure are clustered on chromosomes. Some 850 genes of *Drosophila melanogaster*, when classified by chromosome and by body part affected, including the nature of the change, revealed a tendency, significant at the 1% level, for genes of a kind to occur in the same chromosome. An analysis for 3 classes of genes affecting the eye (colour, shape, texture) and 5 affecting the wing (curvature, length, margin, position, veins) in terms of their positions on the linkage maps showed a significant clustering of genes of similar effect, but when gene sequence was considered without reference to recombination frequencies the genes affecting the same organ were randomly distributed. Thus the clustering of functionally related genes is due entirely to the clustering of all genes. This does not explain, however, the tendency for genes which affect the same structure to occur in a particular chromosome.

Genes affecting the same pathway or the same organ might be expected to be closely linked on functional grounds, in relation to such processes as co-ordinate control of activity or enzyme aggregation. The rarity of such linkage in chromosomal organisms is remarkable and suggests that an opposing force, such as the retention in evolution of particularly favourable combinations of genes of diverse function, may somehow have been of paramount importance.

16. *The hybrid DNA theory of genetic recombination*

§ 16.1 Introduction

When recombination between alleles was first discovered (in *Drosophila* and *Aspergillus*), it was assumed that its mechanism was the same as between different genes. The recombinant individuals, selected because they were wild-type for the character shown by the pair of alleles, usually had one specific new combination of outside marker genes (see § 9.11 and § 13.2), such as is expected with crossing-over. Moreover, reciprocal recombinant genotypes were observed. In *Drosophila* these were from different meioses, but in diploid strains of *Aspergillus*, Pritchard (1955) found several instances of complementary recombinants in the two homologous chromosomes of the diploid, following mitotic recombination between alleles (see § 13.3.) These observations appeared to establish that crossing-over could occur between alleles.

This conclusion was supported by theoretical considerations. The discovery that the linkage map of a series of alleles was collinear with the genetic map of the genes in the linkage group (§ 13.2) suggested that there might be no physical discontinuity between successive genes in the chromosome. If cistrons were segments of a DNA molecule, recognisable from one another merely by their characteristic nucleotide sequence, then it might be expected that the recombination process, whatever its mechanism, would bear no relation to the points of junction of one cistron with another and would be the same irrespective of whether the mutant sites happened to be within the same cistron or not. However, later work, notably with fungi belonging to the Ascomycetes, revealed that recombination between alleles can occur by mechanisms other than crossing-over.

§ 16.2 Recombination in Ascomycetes

As described in § 8.6 with reference to *Neurospora*, the products of meiosis in Ascomycetes are held together in a large cell, the ascus. By isolating the spores from individual asci, detailed information about the process of recombination can be obtained, if there is segregation for linked character-differences. It was by this means that Lindegren (1933) showed that crossing-over occurs between chromatids one from each homologue and that the centromeres segregate at the first division of meiosis (§ 8.6). Subsequently

(Lindegren, 1953), he obtained evidence that in *Saccharomyces cerevisiae* a mutant character, such as inability to ferment α-methyl glucoside, sometimes segregated from its normal allele at meiosis so as to give 3 normal and 1 mutant spores in the ascus, or 1 normal and 3 mutant, instead of the expected 2 of each kind. Lindegren followed Winkler (1930) in calling this phenomenon gene *conversion*. This discovery was so unexpected that it did not gain general acceptance until Mitchell (1955) demonstrated its occurrence with pyridoxin-requiring mutants of *Neurospora crassa*. She made use of linked marker genes on either side of the locus under study, such as were not available in *Saccharomyces*, and so was able to show that the 3:1 ratios at the pyridoxin locus were associated with normal 2:2 ratios for both of the linked character-differences. This established that the 3:1 ratios were due to abnormal segregation during meiosis at a particular mutant site in the chromosome, rather than to abnormal behaviour of whole chromosomes or nuclei.

Many examples of conversion have since been discovered. It is most readily detected with spore characters since the abnormal segregation can then be directly observed in the ascus (Pl. 4(*b*)–(*e*)). Indeed, it had been observed by Zickler (1934) using spore-colour mutants of *Bombardia lunata*, but his observations had been neglected. Olive (1959) examined 2700 asci of *Sordaria fimicola* heterozygous for hyaline *versus* normal black ascospore colour and found 6 asci with 6 black and 2 hyaline spores, and 5 asci with 2 black and 6 hyaline spores. A mutant (*g*) with grey spores showed not only 6:2 and 2:6 ratios for spore colour in heterozygous asci but also 5:3 and 3:5 ratios. This was a remarkable discovery, as it implied that segregation of the character-difference had occurred at the mitosis after meiosis (*postmeiotic segregation*).

Kitani, Olive and El-Ani (1962) discovered another class of asci showing postmeiotic segregation. These asci had 4 black and 4 grey spores but the sequence was abnormal, with half the spores not in pairs. Such arrangements in the ascus can arise from the overlapping of two of the spindles at the third division such that the nuclei pass one another (see Table 8.2, p. 102). However, Kitani *et al.* made use of linked marker genes on either side of the grey spore locus, and this enabled them to distinguish between abnormal behaviour of whole nuclei (spindle overlap) and abnormal behaviour at the site of the spore mutant. The method of distinction is shown in Fig. 16.1 by plus and minus signs. These represent the behaviour of a linked character-difference of the mycelium which could be recognised after dissecting the asci and isolating and germinating the spores. With spindle overlap, the linked character will show the same aberrant sequence as the spore character, but with postmeiotic segregation at the mutant spore site it will not. Moreover, the postmeiotic segregation may give spore sequences such as 1 grey, 4 black, 3 grey, which are unlikely to arise from spindle overlap. Kitani *et al.* examined over 200,000 asci segregating for black and grey spores and found that the 5 classes of aberrant asci resulting from abnormal segregation at the *g* site occurred with widely different frequencies. The numbers in each class expected in a sample of 10^5 asci, according to their data, are shown below the

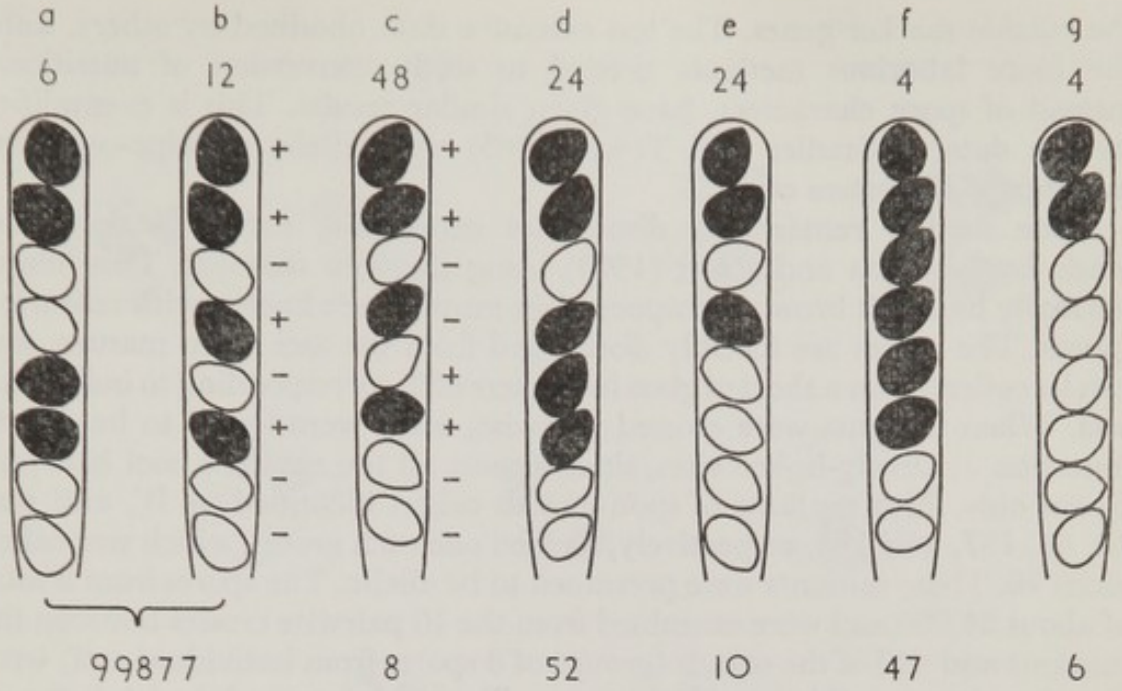


FIGURE 16.1 Asci of *Sordaria fimicola* from a cross between a normal black-spored strain and a mutant with grey spores. The grey spores are shown white in the diagram. The figures below the drawings of asci show the numbers of each kind of ascus expected in a random sample of 100,000 asci, according to counts made by Kitani *et al.* (1962). These numbers refer not only to the spore sequences shown but also to any permutations of them which may arise from variation in the orientation of the appropriate pair of chromosomes on the spindle at any of the nuclear divisions in the ascus, or from the presence or absence of crossing-over in the interval of the chromosome between the gene for spore colour and the centromere. The number of such permutations is shown above the drawings. The plus and minus signs in (*b*) and (*c*) show how a character-difference of the mycelium has segregated. This character-difference becomes evident after the spores germinate, and is due to a gene linked to that for spore colour.

drawings in Fig. 16.1. These numbers include other spore sequences in addition to those shown. The other sequences differ in spindle orientations or in the division of meiosis at which segregation occurred (see § 8.6). The number of alternative sequences which could arise in these ways is shown above the drawings.

A notable feature of the data of Kitani *et al.* is that about 36% of the aberrant asci (classes (*c*)–(*g*) of Fig. 16.1) showed crossing-over between the marker genes on either side of the *g* site. Since these genes were closely linked, with only about 4% of recombination, it is evident that the aberrant segregation at the *g* site shows a highly significant correlation with the occurrence of crossing-over in the immediate neighbourhood. Furthermore, in 42 instances out of 46 the two chromatids involved in the crossing-over were the same as those involved in the aberrant segregation. Nevertheless, a majority of the aberrant asci (64%) showed the parental combinations of

the outside marker genes. The less extensive data obtained by others, using the more laborious methods needed to study conversion of nutritional instead of spore characters, have given similar results. This is exemplified by the data of Stadler and Towe (1963) with allelic cysteine-requiring mutants of *Neurospora crassa*.

Some further remarkable discoveries concerning recombination were made by Lissouba and Rizet (1960) using *Ascobolus immersus*. This fungus normally has dark brown ascospores, but mutants are known with colourless spores. The spores are forcibly discharged from the asci when mature, and can be collected on a sheet of glass in clusters of 8, corresponding to individual asci. When mutants were crossed pairwise, some were found to be due to mutation at closely-linked sites, since almost all the resulting asci had pale spores only. Five mutants of spontaneous origin identified as *W*, and nos. 46, 63, 137, and 188, respectively, formed one such group, which was called series 46. These mutants were presumed to be allelic. The spores from a total of about 84,000 asci were examined from the 10 pairwise crosses between the mutants and 440 of the octads (groups of 8 spores from individual asci) were found to contain wild-type (dark) spores. Two of these octads had 4 dark and 4 pale spores and the remainder had 2 dark and 6 pale. It was evident that the dark spores were arising through recombination, because a total of nearly 10^6 asci homozygous for individual mutants showed none. The frequencies of recombinants from each of the 10 crosses between the different mutants are shown in Fig. 16.2. The recombination frequencies fit a linear map satisfactorily when the mutants are placed in the sequence shown in the diagram, but the larger frequencies are somewhat irregular and tend to be more than the sum of the appropriate smaller ones.

A total of 120 of those octads which contained 2 dark and 6 pale spores were studied further. The spores were germinated and their genotype determined by crossing each with the parental genotypes. The dark spores

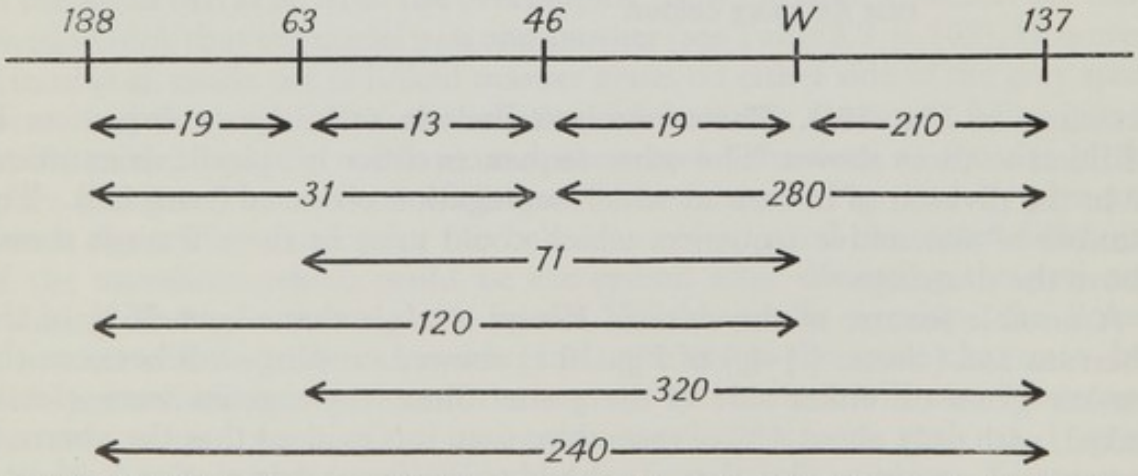


FIGURE 16.2 A recombination map of 5 pale-spored mutants belonging to series 46 in *Ascobolus immersus*, according to the data of Lissouba and Rizet (1960). The reference letters or numbers of the mutants are given above the line. The figures below the line show the frequencies of dark (wild-type) spores per 100,000 progeny ascospores from each pairwise cross.

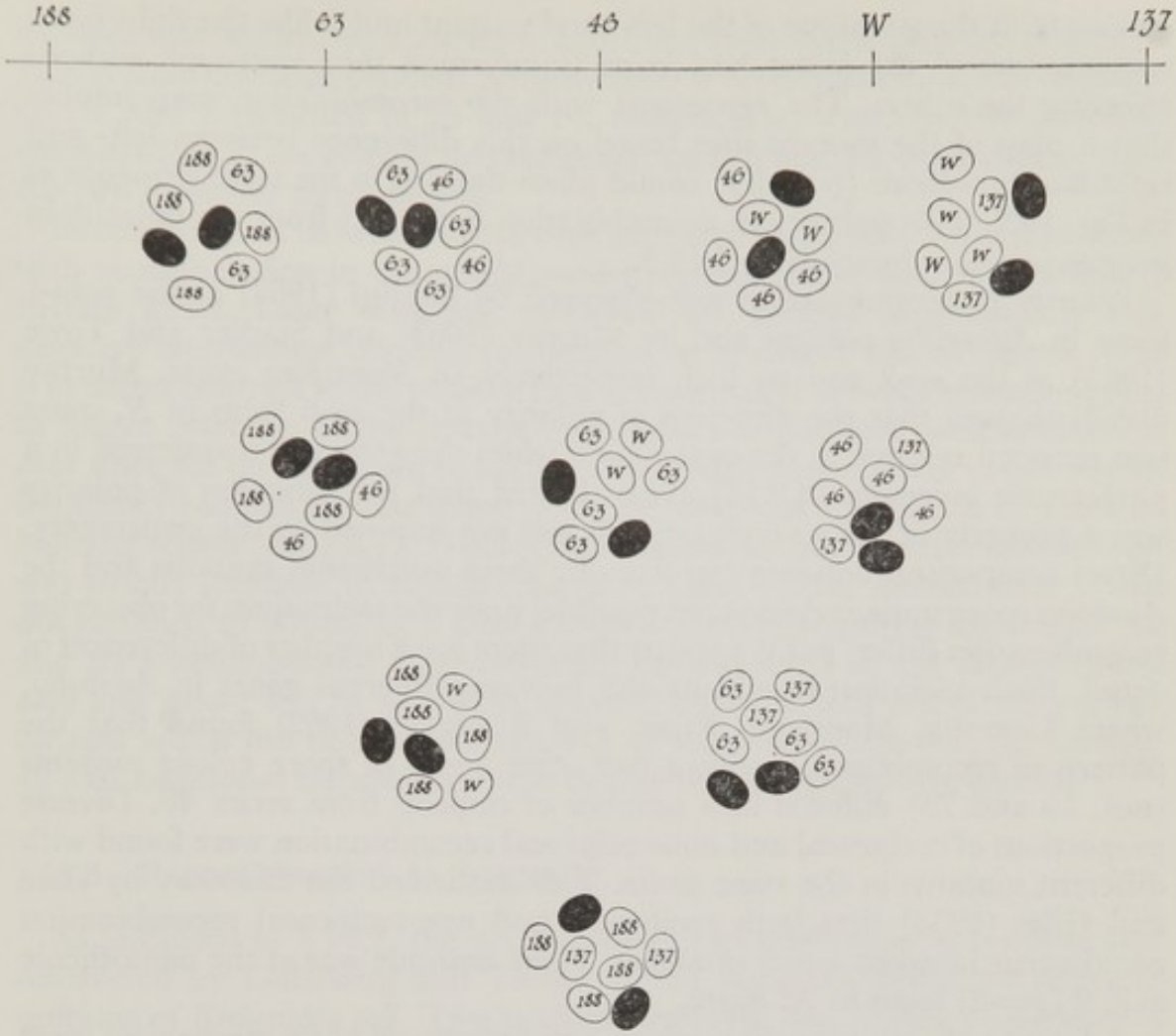


FIGURE 16.3 Diagram to illustrate the genotype of the spores of recombinant asci of *Ascobolus immersus* found by Lissouba and Rizet (1960). At the top is shown the linkage map of 5 pale-spored mutants belonging to series 46, from the data given in Fig. 16.2, and below the map are drawn sets of 8 spores from individual recombinant asci, one from each of the 10 pairwise crosses between the mutants. The black spores are wild-type. The genotype of each pale spore, established from backcrosses to both parents, is shown.

were found to be wild-type as expected. The pale ones invariably gave a few wild-type recombinants with one or other parent but not with both. Double mutant spores would have been revealed, if present, because they would have given no recombinants with either parent, but none was found. Thus all the recombinant (wild-type) spores appeared to have arisen by a non-reciprocal event and not by crossing-over.

Furthermore, in each of these octads 4 pale spores always had the genotype of the parent whose mutant site was to the left on the map, as drawn in Fig. 16.2, and the other 2 pale spores had the genotype of the right-hand parent. For example, in a cross between mutants 188 and *W*, each ascus with 2 wild-type spores was found to have 4 spores of the same genotype as 188 and 2 like *W*, while if *W* was crossed with 137, there were 4 like *W* and 2 like 137 (see Fig. 16.3). It was as if in every tetrad, instead of 2 products of

meiosis with the genotype of the left-hand mutant and 2 like the right-hand mutant, one of the latter had been transformed into wild-type without affecting the others. The agreement with the recombination map implies that a map of the mutant sites based on this difference between left- and right-hand positions (polarity) would place the sites in the same sequence as in Fig. 16.2. This polarity in recombination is distinct from the polarity in gene expression discussed in § 15.3.

Polarity in recombination was observed by Siddiqi (1962) at the *paba*-1 locus in *Aspergillus nidulans* and by Murray (1963) and Stadler and Towe (1963) at the *me*-2 and *cys* loci, respectively, in *Neurospora crassa*. Murray (1968) showed that the direction of polarity at the *me*-6 locus in *N. crassa* was reversed relative to the centromere when the gene was transferred to a paracentric inversion (§ 8.15). This showed that the direction of polarity was a property of the *me*-6 region and was not imposed by the centromere. Direct comparison between the data for these nutritional mutants and the *Ascobolus* spore mutant data is not possible, since the techniques for observing recombination differ, but it appears that there are a number of differences of detail. Such variation is evident also between different genes in *Ascobolus*, where Lissouba, Mousseau, Rizet, and Rossignol (1962) found that the pattern of recombination within two other series of spore colour mutants (nos. 19 and 75) differed in a number of respects from series 46. Diverse proportions of reciprocal and non-reciprocal recombination were found with different mutants in the same series. This confirmed the discovery by Case and Giles (1958) that both reciprocal and non-reciprocal recombination could occur between a pair of alleles. Their example was at the pantothenic acid 2 (*pan*-2) locus in *N. crassa*.

A feature of recombination which any hypothesis of its mechanism must account for is the phenomenon of *negative interference*. A majority of the adenine-independent progeny obtained by Pritchard (1955) from crosses between allelic adenine-requiring mutants of *Aspergillus nidulans* had one specific crossover genotype for linked marker genes on either side of the adenine locus, for example, $y + + bi$ from the heterozygote $\frac{y + \ 8 +}{+ \ 11 + bi}$ (see § 13.2). However, a significant minority showed other combinations of the outside markers ($y + + +$, $+ + + bi$, and $+ + + +$). Giles (1952) had first described this phenomenon with inositol-requiring alleles of *N. crassa*, and it was observed with alleles at the pyridoxin-1 (*pdx*-1) and nicotinic acid 1 (*nic*-1) loci in this species by Mitchell (1956) and St Lawrence (1956), respectively. Among the recombinant progeny which did not require pyridoxin and nicotinic acid, respectively, for growth, not only were all 4 of the genotypes with respect to linked marker genes represented, but one crossover genotype was not markedly the most frequent class such as it would be expected to be with simple crossing-over between alleles. Thus, Mitchell found that 111 pyridoxin-independent progeny consisted of 43 and 28 with one or other parental genotype, respectively, for the outside markers, and 31 and 9 with one or other crossover genotype, respectively. Thus, less than one third of the recombinants could be attributed to a simple crossover

between the alleles. St Lawrence found an even more extreme instance: 72 nicotinic acid-independent progeny from a cross between allelic mutants nos. 1 and 2 consisted of 25 and 14, respectively, with the two parental combinations of the outside marker genes, and 18 and 15, respectively, with the two crossover combinations.

Recombination between alleles thus appeared frequently to be associated with recombination in the neighbouring regions demarcated by the linked marker genes. Later work has confirmed the general occurrence of this negative interference between recombination in closely linked regions. This is in direct contrast to the positive interference found between recombination in longer intervals of the linkage map, where it has been well-established ever since the pioneer work of Muller (1916) that crossing-over in one region frequently reduces the likelihood of its simultaneous occurrence in a neighbouring region (see § 8.7). Pritchard (1955) found that negative interference was strictly localised in extent. However, Calef (1957) showed that it was not confined to alleles. In a cross between adenine-requiring mutant no. 15 and para-aminobenzoic acid requiring mutant no. 1 at the closely-linked *ad*-9 and *paba*-1 loci in *Aspergillus nidulans*, he found that out of 105 recombinants which did not require adenine or *p*-aminobenzoic acid for growth, 49 had additional recombination in one or both neighbouring regions.

§ 16.3 Recombination in bacteria

As indicated in § 10.2, the occurrence of recombination in bacteria was discovered by Lederberg and Tatum (1946) using biochemically deficient mutants of *Escherichia coli*. Two multiple mutants, one having requirements for threonine, leucine and thiamin, and the other for biotin, phenylalanine and cystine, had been obtained by successive treatment with X-rays. The two triple mutants were grown in mixed culture in complete medium containing yeast extract, peptone and glucose, and then suspensions of the washed cells were plated on minimal medium. Wild-type (prototrophic) colonies appeared with a frequency of about 1 per 10^6 cells. This is a much greater frequency than could be attributed to mutation since simultaneous reverse mutation of all three mutants in one or other parent would be required to produce a wild-type colony. Control experiments showed no wild-types when the parents were cultured separately. It was also established by experiment that the prototrophs were not mere mixtures or heterokaryons.

By an extension of the original technique, the recombination of unselected character-differences such as resistance to streptomycin and to particular viruses was studied. Nutritional mutants could be included in the unselected category by plating on a medium with the appropriate supplements. The colonies which developed on this medium were then scored for the unselected character-differences. In this way data on the relative frequencies of recombination between the various mutants were obtained. The recombination frequencies were found to be non-random and to fit a single circular linkage map (Jacob and Wollman, 1958*a*). A similar linkage map has

been obtained from recombination experiments with *Salmonella typhimurium*, and with *Streptomyces coelicolor* (Hopwood, 1967).

The mechanism of transfer of genetic material from cell to cell in *E. coli* has been investigated by a number of authors (cf. Hayes, 1964, 1966*a*, *b*). The details of the process are remarkable. It appears that there is sexual differentiation of cells, the two sexes differing in a number of respects. In particular, they are thought to differ in the charge on the cell surface such that cells of opposite sex may stay in contact if they happen to meet. The male cells are inferred to contain a 'sex factor' which evidently carries the genes for the male characters: it is absent from female cells. This sex factor is capable of replication independently of and more rapidly than the chromoneme*. The sex factor appears to consist of enough DNA to carry 100 or more genes, so it could be regarded as a second chromoneme of about $\frac{1}{50}$ the length of the other. The male cells have one or more special hair-like appendages or pili, with an axial hole 2–2·5 mμ wide which is thought to function as a conjugation-tube. Through this tube one of the editions of the sex factor may be transferred to the female cell, whereupon the latter changes sex. Alternatively, the circular chromoneme of the male cell may be broken open at a particular point, with the sex factor attached to one specific end of it. The chromoneme and sex factor are then transferred to the female cell, with the free end of the chromoneme leading the way. Very frequently transfer is interrupted before completion as a result of random breakage of the chromoneme during transfer, with the result that the sex factor is rarely transferred since it is at the tail. Jacob and Wollman (1958*a*) separated conjugating cells by shaking the culture in a high-speed mixer, and they did this at various time intervals after the start of conjugation, which takes about 100 minutes at 37°C. On the basis of the characters of the male parent which appeared in the progeny, it was possible to find the time at which each gene was transferred. The time sequence map of the genes confirmed the map based on recombination frequencies. Strains of *E. coli* were found to arise, apparently by mutation, in which the sex factor was attached at the other end of the chromoneme, the orientation of the latter during conjugation being reversed so that the sex factor was again at the tail. In other mutants, the circular chromoneme was opened at different points in the linkage group, but the point of opening was constant for any particular mutant.

Jacob and Wollman (1958*b*) coined the term *episome* for structures such as the *E. coli* sex factor and phages, which may exist attached to the chromoneme, whereupon they replicate only when it does, or they may exist unattached, when they replicate independently of and usually more rapidly than the chromoneme. Campbell (1962) proposed that the mechanism of integration of episomes into the host DNA was by crossing-over, the episome having first taken the form of a closed circle of DNA. Excision of the virus from the host DNA would also occur by crossing-over. There is considerable support for this hypothesis, both for the *E. coli* sex factor (Hayes, 1966*a*, *b*) and for phage λ (Signer and Beckwith, 1966). With λ and ϕ80 the evidence includes the demonstration that the linear order of genes in the prophage

* Chromoneme = bacterial (or viral) chromosome—see § 11.1.

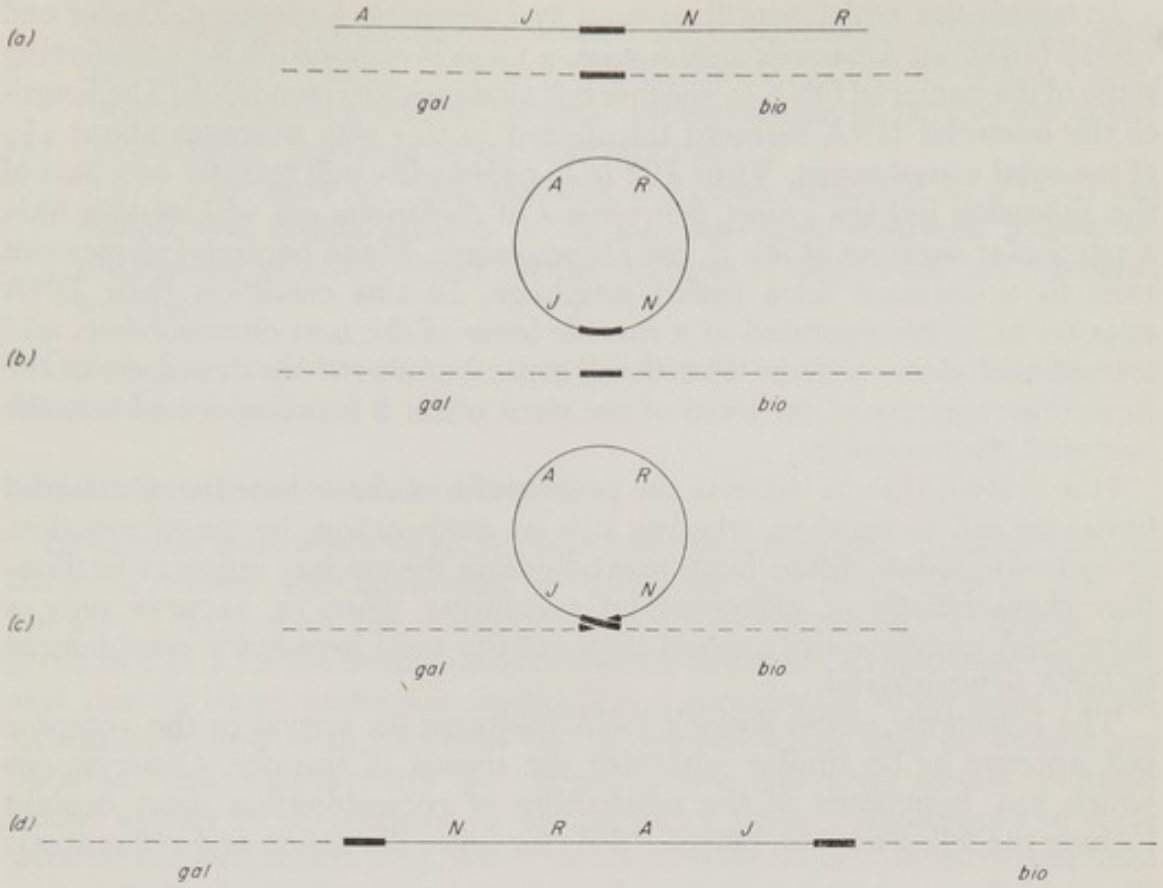


FIGURE 16.4 Linkage maps are shown of virus λ (unbroken line) and part of that of its host, *Escherichia coli* (broken line). The letters *A*, *J*, *N* and *R* identify particular genes in λ, and *gal* and *bio* refer to galactose and biotin genes in *E. coli*. The thick line shows the λ integration-site. (*a*) Free virus, with its linear map. (*b*) Circular form of virus, following fusion of the 'sticky ends' of the DNA (see § 18.9). (*c*) Virus integrated with host DNA, as a result of crossing-over at the integration-site. (*d*) The provirus as part of the same linear structure as the host DNA. The steps in excision of the virus would be represented by the same diagrams in the reverse sequence, that is, (*d*) to (*a*).

is a circular permutation of the order in the free virus, and the finding that bacterial genes on opposite sides of the prophage integration-site are farther apart (as measured by recombination) when the provirus is present (see Fig. 16.4). Direct evidence for specific integrative recombination has now been obtained (see § 16.5).

Two other methods of transfer of hereditary material from cell to cell are known in bacteria, besides conjugation. In genetic transformation, which was discovered by Griffith (1928) in *Diplococcus pneumoniae* (see § 11.2), and has since been shown to occur in *Haemophilus influenzae* and a number of other bacteria, segments of DNA derived from the bacterial cells and equivalent to about $\frac{1}{200}$ of the total hereditary complement, are taken up by other cells of the same or a closely related species. These cells seem to absorb the DNA only during a particular phase of the cycle of cell division.

In *transduction*, which was discovered by Lederberg, Lederberg, Zinder and Lively (1952) in *Salmonella typhimurium*, a virus is responsible for transferring some of the bacterial DNA to another cell along with its own DNA. The length of the bacterial DNA segment transferred in this way averages about $\frac{1}{100}$ of the total complement. Virus *P*22 of *S. typhimurium* will transfer any part of the *Salmonella* linkage group, but virus λ of *Escherichia coli* will transfer only a particular segment of the *E. coli* chromoneme. Many bacterial viruses can exist in a dormant form called prophage. In this condition their DNA appears to be incorporated at a specific locus of the host chromoneme, and to replicate along with it. The *E. coli* genes transferred by virus λ are in the immediate vicinity of the locus of the virus when it is incorporated into the bacterial chromoneme.

It is evident that in bacteria the process of transfer of hereditary material from one cell to another, whether it is by conjugation, by transformation, or by transduction, differs fundamentally from the nuclear fusion at fertilisation characteristic of chromosomal organisms, since in bacteria only a part—and usually quite a small part—of the total hereditary complement of DNA is transferred.

The behaviour of the donor's DNA fragment on arrival in the acceptor cell appears to be similar whatever the means of transfer. Observations which any hypothesis of the mechanism of recombination must explain include the following (see Hayes (1964) for fuller discussion and references):

(*a*) A transferred gene from the donor may function at once in the acceptor cell, for example, in conferring resistance to streptomycin on a previously sensitive cell, but it does not follow from this that the progeny of this cell will necessarily inherit the donor character. This phenomenon is particularly well-known with transduction in *Salmonella* and is called *abortive transduction*. It appears that the donor DNA fragment fails to replicate and is therefore inherited by one daughter-cell only. When a donor character does become fixed in the progeny of the acceptor cell, it is evident that recombination has occurred such that the donor allele has become incorporated into the chromoneme of the acceptor cell in place of the acceptor allele.

(*b*) By labelling the donor DNA of conjugating *E. coli* with radioactive phosphorus (^{32}P), Jacob and Wollman (1958*a*) showed that, probably before chromonemal replication and certainly less than an hour after transfer, genetic information in the donor fragment has begun to be conveyed to material free of ^{32}P. This was shown by a slowing down in the rate of loss of recombinants through radioactive breakdown of the ^{32}P.

(*c*) Isolation of the individual cells formed at each division after conjugation has shown that segregation of a parental character-difference may occur as late as the ninth division.

(*d*) Reciprocal differences in recombination frequency, depending on which parent is the donor, have been reported both with transformation and transduction.

(*e*) Isotope labelling experiments have established that both with conjugation (Siddiqi, 1963) and transformation (Fox and Allen, 1964), recombination can involve physical incorporation of part of the donor chromoneme into that of the acceptor cell.

(*f*) The work of Yanofsky and associates discussed in § 14.13 has shown that recombination in *E. coli* can occur between individual nucleotides of DNA. Some further features of their work are discussed in § 16.6.

Other experimental results from studies on recombination in bacteria are discussed in § 16.5 and § 16.6.

§ 16.4 Recombination in viruses

As already mentioned (§ 13.4), the occurrence of genetic recombination in bacterial viruses was discovered by Delbrück and Bailey (1947) and by Hershey (1947) with the *T*-even viruses of *Escherichia coli*. Evidence was given in § 11.3 for the belief that only the DNA of the virus enters the host cell, and that it there undergoes replication a number of times prior to the formation of new virus particles, 100 or more of which may be formed per bacterial cell. Opportunity for recombination following mixed infection would appear to be confined to the period of DNA replication. The early data on recombination frequencies obtained with these *T*-even viruses from crosses (mixed infections) such as those described in § 13.4, were found to fit linear linkage maps, and more recently these maps have been shown to be part of a single circular linkage map (Epstein *et al.*, 1964—see Chapter 18). This does not necessarily mean that the DNA is in a circular molecule: it seems in fact to be linear with terminal redundancies, that is, the two ends have the same nucleotide sequence; and different individuals have the ends in different places on the map, so that they are circular permutations of one another (see Streisinger, Emrich and Stahl, 1967).

Outstanding discoveries which provide information about the mechanism of recombination in bacterial viruses include the following. Hershey and Rotman (1949) found that if the host is simultaneously infected with 3 different strains of virus *T*2, some of the progeny may have characters from all 3 parents. This would be accounted for if opportunities for recombination occurred repeatedly during the successive replications of the DNA. Hershey and Chase (1952*a*) discovered that about 2% of the virus progeny, following mixed infection by strains with clear and with turbid plaques, had mottled plaques. They established that the mottled plaques were initiated by virus heterozygous for the pair of alleles. It appeared that the heterozygous region was quite short. For every locus studied, a similar frequency (about 2%) of such heterozygotes was found. Using several genetic markers simultaneously, Levinthal (1954) found that heterozygosity at a particular locus was usually associated with recombination between markers situated at a distance on either side, suggesting that the origin of the heterozygosity is related to that of the recombinants. Chase and Doermann (1958) found that with closely-linked mutants of virus *T*4, recombination in one interval

appeared to favour recombination in a neighbouring one, or in other words, there was 'negative interference' over short intervals of the linkage map, comparable to that known in chromosomal organisms (see § 16.2).

Meselson and Weigle (1961), by an ingenious experiment, showed that recombination in virus λ of *Escherichia coli* could occur independently of replication of the chromoneme. A mixed infection of the host was made using two strains of the virus which differed from one another in two linked mutant characters concerning the morphology and the clarity of the plaque. As a result of multiplication in the host when cultured on appropriate media, the parental viral strains also differed in the atomic weight of their carbon and nitrogen ($^{13}C\ ^{15}N$, and the normal $^{12}C\ ^{14}N$, respectively). The progeny virus particles, resulting from inoculation of normal $^{12}C\ ^{14}N$ *E. coli* with the mixture, were centrifuged in caesium chloride solution to give a density gradient. The contents of the centrifuge tube were then removed drop by drop from the end, and the number of virus particles in each drop estimated by diluting and plating on the host. The genotype with respect to the two parental character-differences was observed for a random sample of the progeny particles in each drop. It was found that the progeny, whether of parental or recombinant genotypes, were concentrated at three positions in the centrifuge tube corresponding to heavy ($^{13}C\ ^{15}N/^{13}C\ ^{15}N$), half-heavy ($^{13}C\ ^{15}N/^{12}C\ ^{14}N$), and light ($^{12}C\ ^{14}N/^{12}C\ ^{14}N$) DNA, indicating 0, 1 (or more), and 2 (or more) replications of originally heavy DNA, respectively (cf. Fig. 12.5). The significant discovery was the occurrence of recombinants with an unreplicated ($^{13}C\ ^{15}N/^{13}C\ ^{15}N$) chromoneme.

Kellenberger, Zichichi and Weigle (1961) obtained similar results using a slightly different technique which did not involve culturing on 'light' or 'heavy' bacteria. As genetic markers they used mutants with abnormal DNA contents, which could be separated by density-gradient centrifugation, and as nucleotide-chain markers they used radiophosphorus (^{32}P) and followed the distribution of its radioactivity.

Meselson (1964), in further experiments with virus λ labelled with heavy isotopes, has found some indication that a small amount of DNA is removed and resynthesized in the formation of recombinant molecules.

Further studies on recombination in λ are described in § 16.5.

§ 16.5 Hypotheses to explain recombination

Several hypotheses have been put forward to explain the remarkable series of discoveries concerning recombination described in the previous sections. Giles (1952) and Pritchard (1955) suggested that multiple crossover events might be responsible for the phenomena they had observed. If crossovers occurred in clusters, this would explain the negative interference within short regions. Pritchard attributed the positive interference over longer intervals to spacing of the regions where pairing of homologous chromosomes was sufficiently effective to allow crossovers to occur. The effective pairing segments would be variable in position, but rarely close together.

By itself, this hypothesis was clearly inadequate to explain non-reciprocal recombination. Lederberg (1955) suggested that recombination in bacteria could be related to the process of replication of the hereditary material, which might be copied first from one parent and then, further along the chain-molecule, from the other parent. Freese (1957) applied this model to chromosomes. It was suggested that if a daughter-chromatid were formed by a 'conservative' replication process, that is, by the copying of the hereditary material of a parent chromosome so as to leave the latter intact, then a process of copy-choice or switch-synthesis might operate when two homologous chromosomes were closely associated with one another. If the synthesis of the two daughter-chromatids, one from each parental chromosome, was not quite synchronised, both might be copied over a short interval from the same parent, thereby giving rise to a 3:1 ratio within a tetrad at a mutant site. This explanation of non-reciprocal recombination was combined with the multiple crossover idea by postulating that the switching back and forth between the parental templates during the synthesis of the daughter-chromatids occurred several times. This hypothesis explained a number of the observations on recombination, but it suffered from several serious defects:

(1) The conservative method of replication of chromosomes was in conflict with the evidence that both DNA and chromosomes replicate semi-conservatively (see Chapter 12). It was therefore incorrect to speak of parent- and daughter-chromatids, since the DNA of each chromatid is half old and half new.
(2) The copy-choice hypothesis predicts that successive crossovers along a chromosome arm will involve the same two chromatids (the daughters, not the parents), but the pair of chromatids involved in each crossover appear to be chosen at random, or nearly so (see § 8.8).
(3) Replication of both the DNA and the protein of the chromosome appears to take place before the homologous chromosomes start to associate at the zygotene stage of meiosis (see § 11.5 and Pl. 2(*a*)). Rossen and Westergaard (1966) showed by Feulgen micro-spectrophotometry that in the fungus *Neottiella rutilans* the premeiotic DNA synthesis actually took place before nuclear fusion, and Sueoka, Chiang and Kates (1967) have made a similar discovery with *Chlamydomonas reinhardi* (see § 17.9). Henderson (1966), working with males of the locust *Schistocerca gregaria*, found by combined tritium labelling (to observe DNA synthesis) and high temperature treatment (to modify chiasma frequency), that chiasma formation occurred several days after the premeiotic DNA synthesis.
(4) Isotope labelling experiments have shown that recombinants, both in bacteria and their viruses, may contain parental atoms (§ 16.3 and § 16.4).
(5) Postmeiotic segregation (cf. Pl. 4(*e*)) was unexplained.
(6) The inequality in the frequency of 6:2 and 2:6 ratios of black:grey spores found by Kitani *et al.* with the *g* mutant of *Sordaria fimicola* was unexplained.

(7) Recombination must occur with molecular precision, such that nucleotides are rarely duplicated or omitted*, but it is not clear how this would be achieved with a switching of copying from one template to another.

Most of these defects in the copy-choice hypothesis can be avoided by making suitable modifications or additional postulates, and numerous highly elaborate theories have been put forward. However, to account for both postmeiotic segregation and 6:2 and 2:6 ratios shown by the same mutant, such as Kitani *et al.* have found with the grey-spored mutant of *S. fimicola*, requires an intricate and implausible model. It appears that none of the variants of the copy-choice hypothesis will provide a satisfactory explanation for all the observations, although the model proposed by Stadler and Towe (1963) accommodates a majority of them.

Taylor (1965) obtained the first direct evidence that crossing-over at meiosis takes place by breakage and exchange. He used ^{3}H-thymidine labelling of the chromosomes in males of the grasshopper *Romalea microptera*. This discovery, however, does not eliminate the possibility that copy-choice might also be involved in recombination.

I proposed (Whitehouse, 1963) a model based on breakage and rejoining of chromatids. It was assumed that replication of chromosomes is completed prior to the occurrence of recombination, and that during the zygotene or pachytene stages of meiosis, when the homologous chromosomes are associated in pairs, recombination occurs at a number of points along the length of the chromosome, and that one chromatid from each chromosome, selected at random, is involved each time. It was also assumed that the reconstruction of DNA molecules in new ways, following breakage, took place not by an end-to-end association of broken chains but by a lateral association of complementary segments from homologous regions, to give *hybrid DNA*. End-to-end joining would appear readily to allow deficiencies or duplications of nucleotides to arise, whereas lateral association would automatically give great precision to the recombination process. Furthermore, it has been shown by Marmur and Lane (1960) that complementary nucleotide chains of DNA will associate spontaneously *in vitro* under suitable conditions to give the duplex molecule. It therefore seemed likely that re-assembly of broken molecules might occur *in vivo* in a similar way. If, in the process of crossing-over, the two DNA molecules assumed to be involved were broken one chain at a time, re-association in new ways could occur before the second chains were broken. This might have a selective advantage by reducing the likelihood of complete breakage without rejoining. Failure of rejoining would lead ultimately to loss of the acentric part of the chromosome, with lethal consequences for that cell lineage (cf. § 9.8). If the initial breakage was in homologous regions of complementary chains, one from each molecule, crossing-over could occur by a succession of steps involving chain separation, synthesis of new chain segments, and base pairing of complementary segments from the two molecules.

Holliday (1964) independently proposed a different hybrid DNA model.

* Evidence for occasional duplication or omission of nucleotides is discussed in § 16.6.

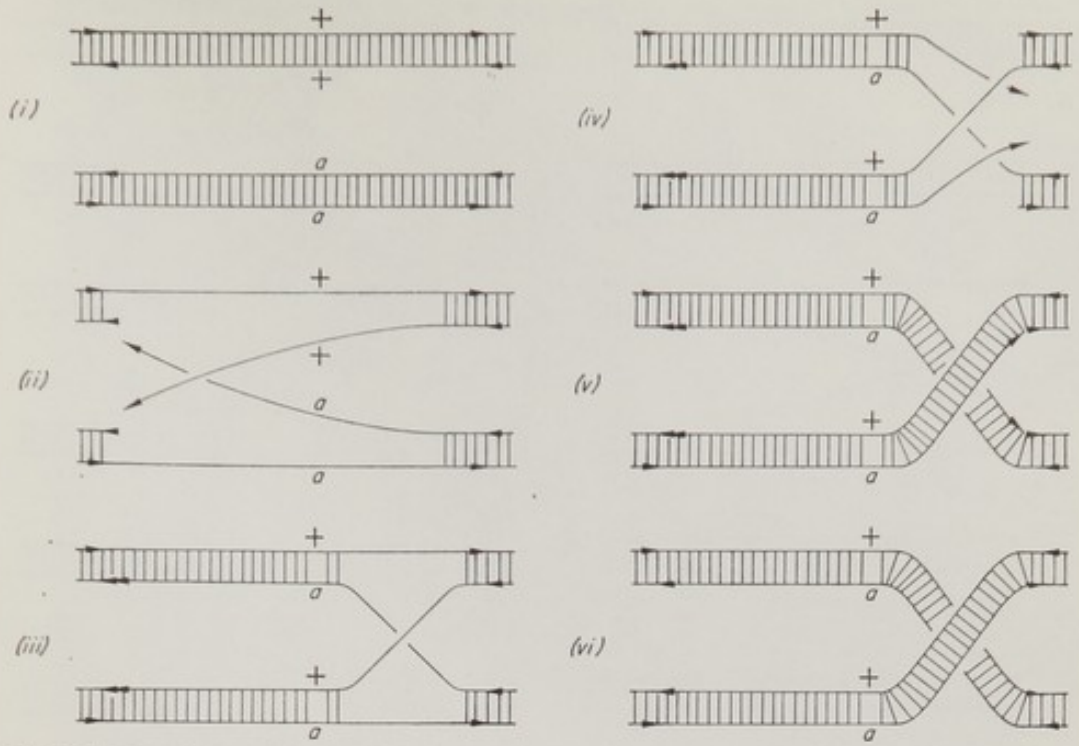


FIGURE 16.5 The mechanism of crossing-over according to the hybrid DNA model of Holliday (1964). The lines with arrow-heads represent nucleotide-chains, with the arrows showing the direction of the phosphate-sugar backbones. Transverse lines indicate hydrogen bonding between complementary bases. The letter *a* indicates the site of a mutation, and the plus sign its normal allele.

He suggested that the initial breakage was in chains of the same polarity. Crossing-over could then take place merely by chain separation followed by annealing between the complementary chains from the two molecules, without the necessity for any DNA synthesis. Breakage and exchange of the other two nucleotide-chains would complete the process (Fig. 16.5).

Without specifying the sequence of events in detail, Meselson (1965) has also suggested that recombination involves pairing between complementary DNA chains of different parentage. He favours a model in which recombination may be associated with the excision of chain segments and their resynthesis along the complementary chain derived from the other parent.

In order to account for polarity in recombination, it appears necessary to postulate the existence of discontinuities at intervals along the chromosome in the pattern of recombination. Hastings and Whitehouse (1964) suggested that the initial points of breakage of the DNA chains could occur only at fixed positions which an enzyme could recognise. The steps in the process of crossing-over according to this model are illustrated in Fig. 16.6. Only 2 of the 4 chromatids are shown, as the other 2 do not take part (although they may participate in another crossover further along the chromosome). No attempt has been made to show the spiral coiling. In Fig. 16.6(ii), breakage of complementary chains has occurred at corresponding positions, and the chains have uncoiled. In (iii), synthesis of nucleotide-chains has

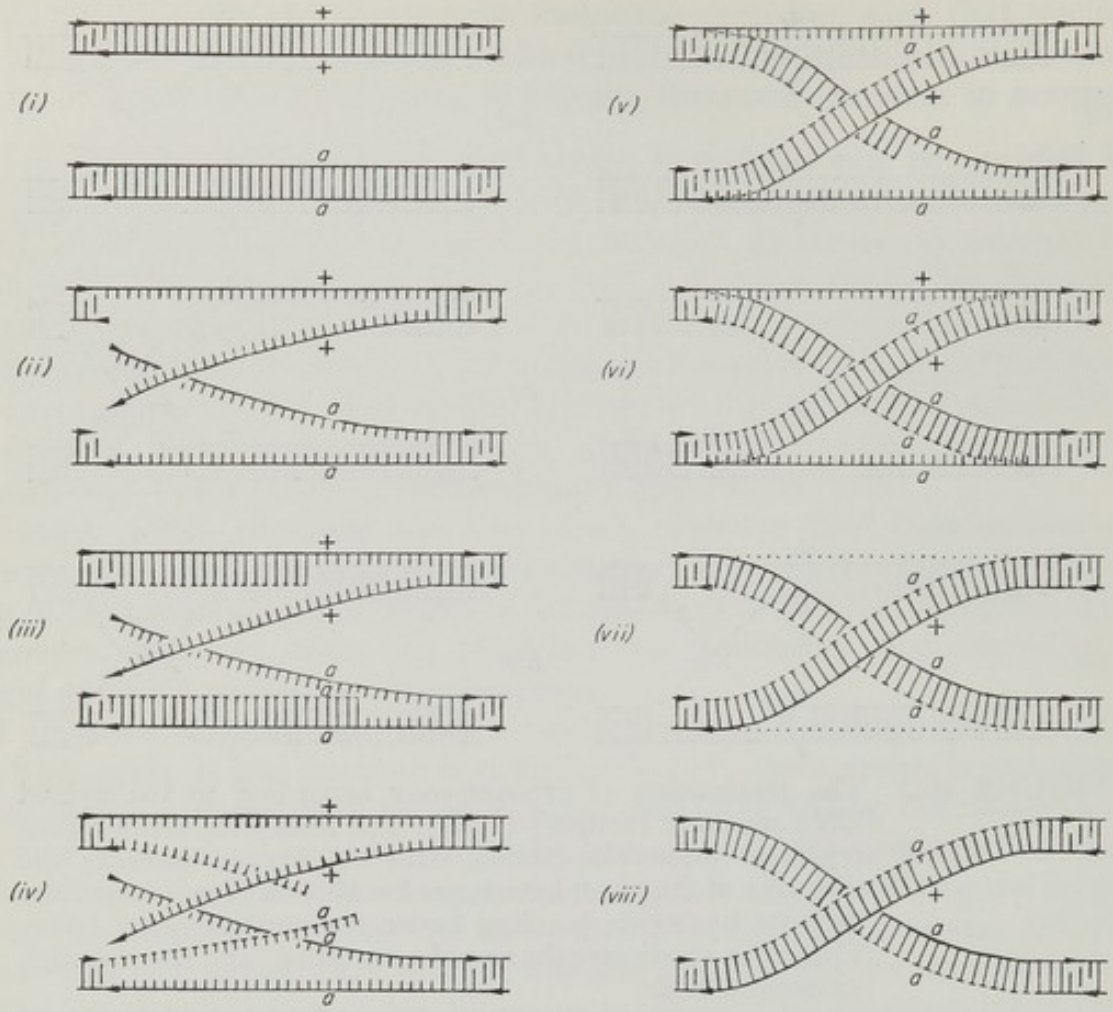
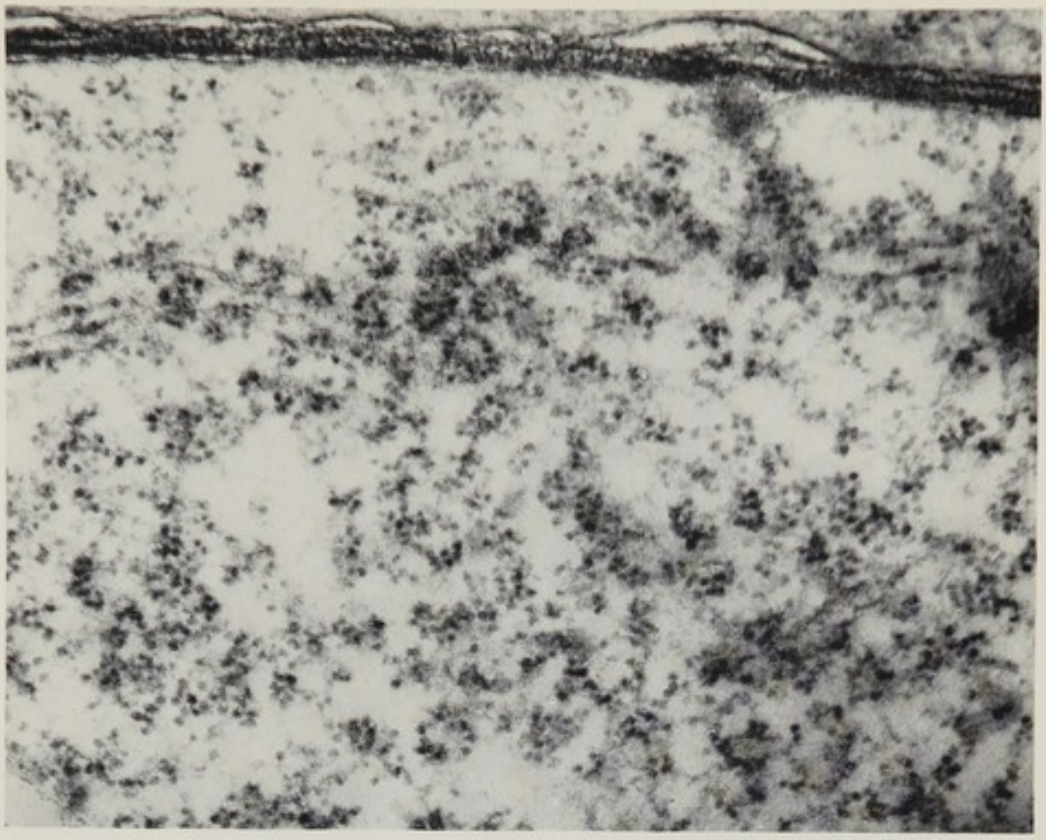


FIGURE 16.6 The mechanism of crossing-over according to the hybrid DNA model proposed by Whitehouse and Hastings (1965). The lines with arrow-heads represent nucleotide-chains, with the arrows showing the direction of the phosphate-sugar backbones. Broken lines represent newly-synthesised chains, and dotted lines chains which are breaking down. The arrow-heads are placed at the points where breakage of the chains can occur enzymatically. Transverse lines extending from one nucleotide-chain to another indicate hydrogen bonding between complementary bases. The letter *a* indicates the site of a mutation, and the plus sign its normal allele.

occurred alongside both of the unbroken chains and complementary to them. The newly-synthesised chains are shown by broken lines, and need not be of equal length. In (iv), the newly-synthesised chains have dissociated from their templates, and in (v) they have associated with the complementary broken chains from the other molecule. The final stages of crossing-over involve the filling of any gaps in the duplex crossover molecules with nucleotides complementary to the other chain (Fig. 16.6(vi)), and the breakdown of the unpaired non-crossover chains (vii) to give the completed crossover (viii). It will be noticed that there is no net synthesis of DNA.

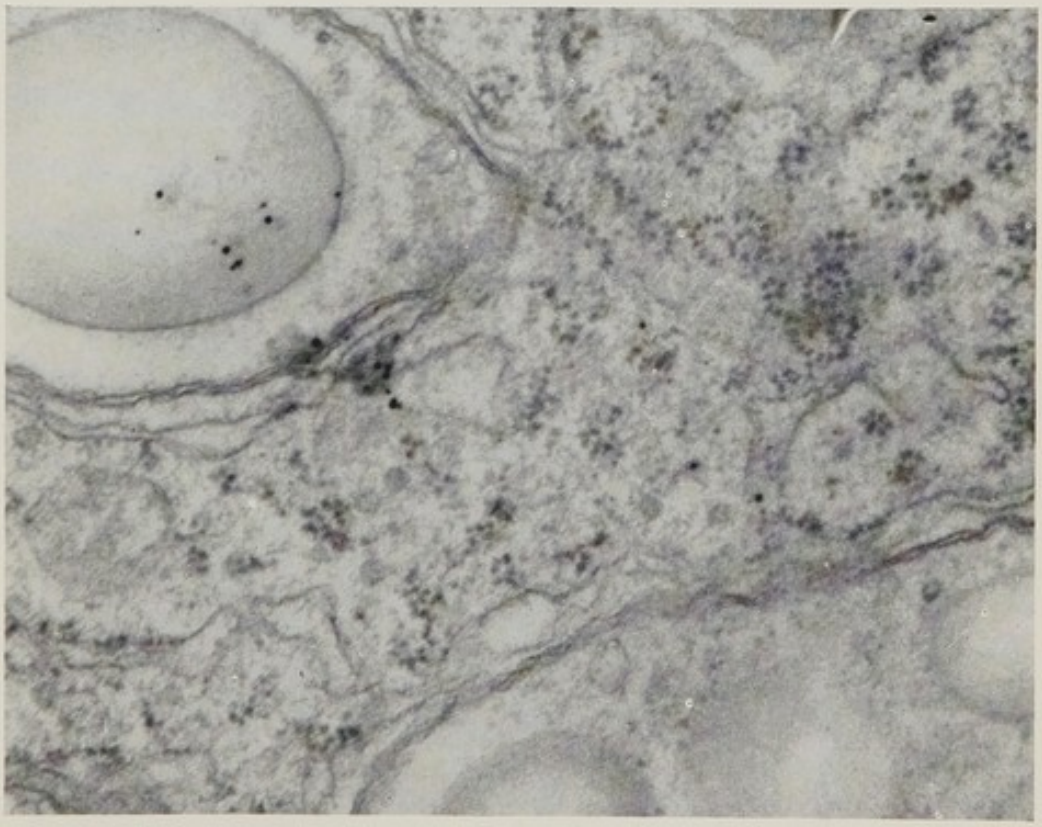
There is experimental evidence for the occurrence of a limited amount of DNA synthesis at the pachytene stage of meiosis, such as this hypothesis

PLATE 5 Electron micrographs of polyribosomes (see §14.11).



(*a*) Part of a sporogenous cell in a young anther of *Helleborus foetidus* (×63,000). Some of the ribosome aggregates are associated with the endoplasmic reticulum. At the top there is a cell-wall.

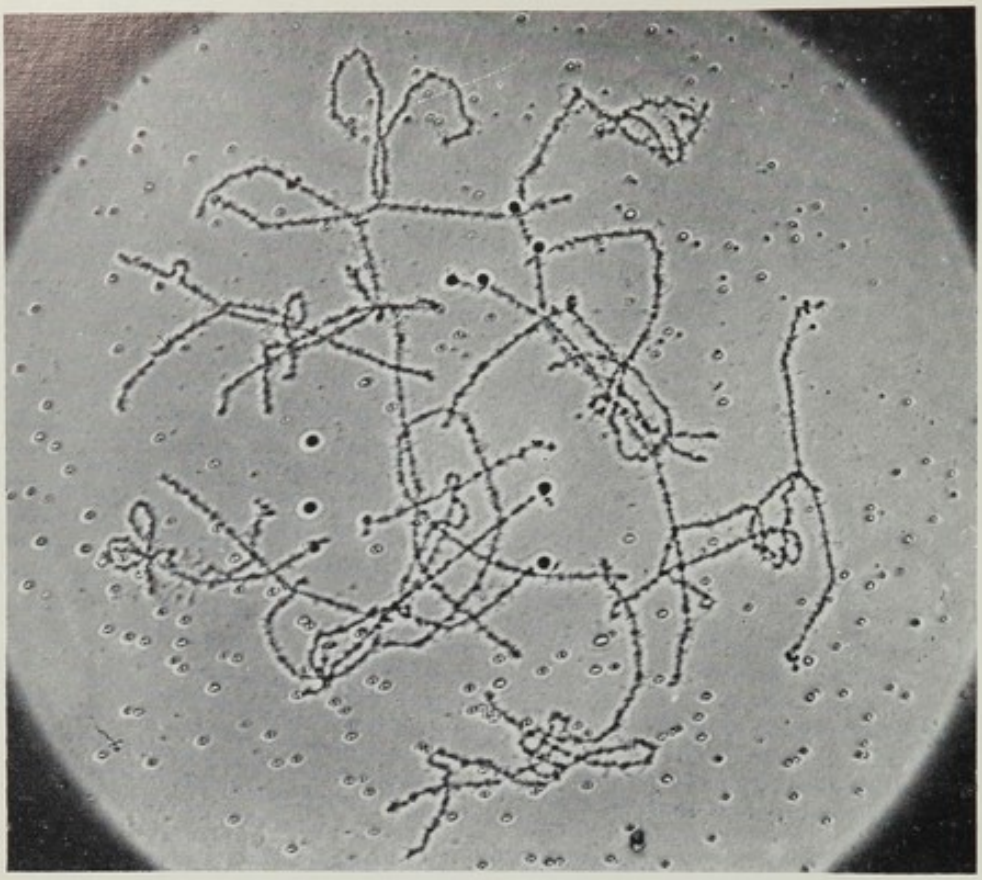
(*Courtesy of P. Echlin*)



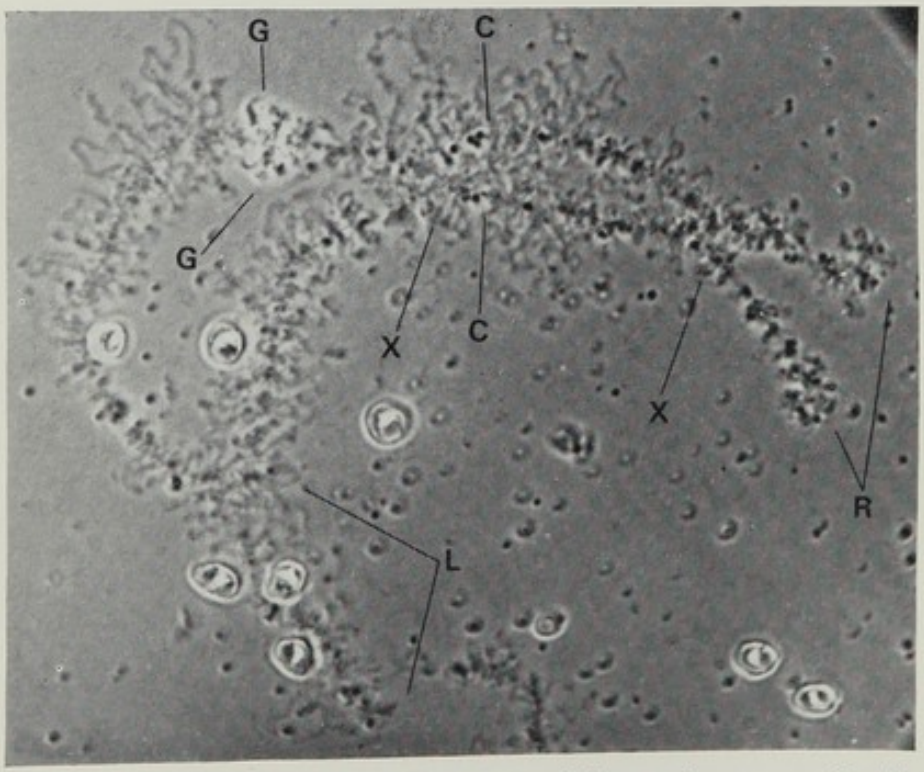
(*b*) Curvilinear configurations of polyribosomes in the cytoplasm of pollen-grains of *Endymion nonscriptus* (×38,000). Starch grains and endoplasmic reticulum can be seen.

(*Courtesy of R. E. Angold*)

PLATE 6 Lampbrush chromosomes from oocytes at the diplotene stage of meiosis in subspecies *carnifex* of the newt *Triturus cristatus* (see §17.6). The highly refractive spherical objects are free nucleoli (compare §18.9). (*Courtesy of H. G. Callan*)



(*a*) Phase-contrast photograph of the entire chromosome complement consisting of 12 bivalents (×60). Each has 2 or 3 chiasmata.



(*b*) Bivalent XII showing the lateral loops in more detail (×300). C = Centromere. G = Giant fusing loop. L = Left-hand end. R = Right-hand end. X = Chiasma. The individual from which this chromosome-pair was obtained was heterozygous for the presence and absence of loop G. In spite of a proximal chiasma, the two chromatids with this loop remain paired, and likewise with the two without the loop (see §8.13).

demands. Wimber and Prensky (1963), using tritiated thymidine, have shown autoradiographically that some DNA synthesis occurs at this stage of meiosis in *Triturus viridescens*. Hotta, Ito and Stern (1966) found from studies of the uptake of radioactive phosphorus (^{32}P) that about 0·3% of the total DNA of *Lilium longiflorum* and *Trillium erectum* was synthesized at the zygotene and pachytene stages of meiosis in the anthers (see §§ 18.8 and 9).

If crossing-over is associated with hybrid DNA formation, as this hypothesis supposes, the site of a mutation will sometimes happen to lie within the hybrid region, giving rise to *heterozygous DNA*. The consequences of such heterozygosity in the molecule are discussed in the next section.

Hybrid (heteroduplex) DNA formation as a result of base-pairing between complementary nucleotide-chains derived from two different molecules, would also appear to fit many of the observations on recombination in bacteria and viruses described in § 16.3 and § 16.4. Hypotheses to this effect were put forward by Szybalski (1964) and Meselson (1964). Much support for heteroduplex models in bacterial and viral recombination has since been obtained. Fox and Allen (1964) working with *Diplococcus pneumoniae* and Bodmer and Ganesan (1964) with *Bacillus subtilis* showed by experiments using heavy isotopes that genetic transformation involved the physical incorporation of segments of donor DNA into the DNA of recipient cells, and that single-chain segments of donor DNA were covalently bound within recipient DNA molecules. Gurney and Fox (1968), in further experiments on transformation in *D. pneumoniae* using heavy isotopes, obtained DNA that was both physically and genetically hybrid, and they showed that either chain of the duplex could participate in the breakage and rejoining mechanism which effects transformation. Anraku and Tomizawa (1965) obtained evidence, by caesium chloride density-gradient centrifugation of labelled DNA from mutants defective in particular genes, that in recombination in virus *T*4 of *E. coli* the heteroduplex is held together at first only by hydrogen-bonding between the parental chains, but that after single-chain gaps have been filled by DNA synthesis the heteroduplex becomes covalently bonded. Weiss and Richardson (1967) purified an enzyme, DNA ligase, from *E. coli* infected with *T*4. This enzyme catalyzes the covalent joining of two segments of a broken chain of a DNA duplex to give a phosphodiester bond, and is coded by gene 30 (Fig. 18.3) in *T*4 (Fareed and Richardson, 1967). Similarly, Oppenheim and Riley (1967) showed that, following conjugation in *E. coli*, the covalent joining of donor and recipient DNA segments takes place after non-covalent bonding.

The occurrence of recombination in bacteria and viruses by breakage followed by rejoining by hybrid DNA formation has thus been established. There is no evidence, however, for polarity in recombination in these organisms, such as is known in fungi; this probably means that, in general, bacterial and viral recombination can be initiated at any point in the DNA rather than at specific ones. This conclusion is in agreement with Kozinski, Kozinski and James' (1968) belief that recombination in *T*4 is initiated by single-chain cuts in the DNA caused by an enzyme coded by the virus.

Crossing-over at a specific site is required, however, for the insertion (integration) and removal (excision) of an episome from its specific attachment-site on the host chromoneme (§ 16.3). Signer and Beckwith (1966) found that virus $\phi 80$ directed the synthesis of a special system which promoted recombination with the $\phi 80$ integration-site on the *E. coli* chromoneme. Echols, Gingery and Moore (1968) and Weil and Signer (1968) identified a λ gene, *intA*, which is specifically concerned with recombination at the site of insertion of λ into the *E. coli* chromoneme, but does not affect recombination within λ except at the integration-site. This is in contrast to another λ gene, *red*, mutants of which are defective in λ recombination but capable of integration with and excision from the host DNA, even when the host is recombination-deficient (*rec*). Possible interactions between the host (*rec*), viral (*red*) and host-viral (*int*) recombination systems have not yet been analysed in detail. The sequence on the λ linkage map of the *red* and *int* genes is: integration-site, *intA*, *red*, *N*, c_I (see Fig. 16.4).

The specificity of control of episome integration and excision, as well as the reciprocal nature of the exchange, resemble crossing-over in chromosomal organisms (see § 16.7). Indeed, Cross and Lieb (1967) proposed that excision of λ prophage from the *E. coli* chromoneme took place in the same way as Holliday (1964) had suggested for crossing-over in chromosomal organisms, except that Cross and Lieb favoured the exchange of chains to give regions of hybrid DNA occurring by the looping-out of unbroken chains, with the breakage occurring subsequently.

§ 16.6 Correction of anomalies in DNA

Kelner (1949) showed that the lethal effect of ultraviolet light on the conidia of *Streptomyces griseus* could be reversed by subsequent treatment with visible light. This process of photo-reactivation has since been found to apply to a wide range of organisms, although not to all, and to diverse effects of ultraviolet light on them. Numerous observations pointed to an effect of the ultraviolet light on DNA as the primary source of the changes induced. *Haemophilus influenzae* shows no photoreactivation, but Rupert, Goodgal, and Herriott (1958) demonstrated that a cell-free extract from *Escherichia coli*, which is a photoreactivable species, will reactivate the DNA of *H. influenzae* in the presence of visible light. This was shown by an increased ability of DNA from streptomycin-resistant bacteria to transform streptomycin-sensitive cells. The ultraviolet treatment reduced the transforming ability to 1% of the original, and illumination with visible light in the presence of the *E. coli* extract raised it again to between 10 and 50% of the original value. Rupert (1960) isolated an enzyme from *Saccharomyces cerevisiae* which had a similar effect to the *E. coli* extract. It is evident that a number of organisms possess an enzyme system capable of bringing about 'repair' of 'damage' to DNA caused by ultraviolet light. Beukers and Berends (1960) showed that u.v. irradiation of thymine in a frozen aqueous solution leads to the formation of thymine dimers. It was provisionally suggested that these had carbon–carbon bonds between atom 4 of one thymine and atom 5 of

the other, and *vice versa*. Wacker, Dellweg, and Weinblum (1960) studied the effect of ultraviolet irradiation of the DNA from *Streptococcus faecalis* labelled with ^{14}C in the thymine, and concluded that the mutagenic and lethal effects of ultraviolet light on cells were likely to be due to its chemical effect on thymine in DNA. Wulff and Rupert (1962) showed that the enzymic repair in the presence of light occurs by specific cleavage of the thymine dimers *in situ*, that is, by a reversal of the ultraviolet-induced process. Setlow, Boling and Bollum (1965) discovered that ultraviolet light caused the formation of cytosine dimers in DNA as well as thymine dimers, and that the photoreactivating enzyme splits both kinds.

Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964*a*) showed that an ultraviolet-resistant strain of *Escherichia coli* had the ability to remove thymine dimers from its DNA, whereas a particular ultraviolet-sensitive strain lacked this capacity. This reaction did not require visible light. It appeared that a specific gene was responsible. Boyce and Howard-Flanders showed that the dimers were extracted as a whole, including the intervening phosphodiester bond, and that nucleotides were then inserted into the excised region by complementary pairing with the intact opposite chain, and the broken phosphodiester backbone was rejoined. It is of particular significance that the repair mechanism appears to make use of the base sequence of the complementary chain. Extending these studies, Boyce and Howard-Flanders (1964*b*) found that in *E. coli* the repair in the dark of ultraviolet-irradiated DNA is controlled by 3 genes (*uvrA*, *uvrB* and *uvrC* for ultraviolet repair) which are widely spaced in the chromoneme. Mutation of any one of these genes produced an ultraviolet-sensitive mutant unable to excise thymine dimers, and these mutants showed increased sensitivity to the lethal effects of mitomycin *C*. It was inferred that damage to DNA caused by mitomycin *C* is repaired by the same molecular mechanism as ultraviolet damage, and that the enzymes recognise distortions of the phosphodiester backbones of the nucleotide chains, rather than the precise chemical form of the defective bases.

Pettijohn and Hanawalt (1964) discovered that repair-replication of DNA took place after *E. coli* had been irradiated with ultraviolet light. From caesium chloride density-gradient studies they showed that 5-bromouracil (a density label) was incorporated into short segments of single chains. They concluded that damaged regions of single chains were excised and replaced by normal nucleotides using the undamaged chain as template. The repair of a partly single-chain DNA molecule might come about through the action of the DNA polymerase isolated by Kornberg and associates from *E. coli* (see § 12.4), since Richardson, Inman, and Kornberg (1964) have demonstrated the *in vitro* ability of this enzyme to restore the full double-chain structure after limited digestion with exonuclease III. The latter enzyme will act as a phosphatase and hydrolyse 3′-phosphate groups at the end of a DNA nucleotide chain of high molecular weight, and will then act as an exonuclease to bring about stepwise hydrolysis from the 3′-hydroxyl end, releasing 5′-nucleotides (cf. Richardson, Schildkraut, and Kornberg, 1964).

Howard-Flanders and Boyce (1966), as a result of their studies with the *uvr* mutants, believe that the repair of DNA in the dark occurs in four steps: (1) excision by an endonuclease of a trinucleotide containing the pyrimidine dimer or other damaged nucleotides as a unit, (2) widening of the gap for a distance estimated to average nearly 500 nucleotides by an exonuclease acting on a free end of the chain, (3) repair replication by a DNA polymerase, using the intact chain as template, to fill the gap, and (4) joining the phosphodiester backbone after the last nucleotide has been inserted: an enzyme, DNA ligase, which catalyzes this reaction has now been isolated from *E. coli* as well as from virus *T*4-infected cells (§ 16.5). The exact molecular steps of this repair process are uncertain, however; for instance, does the polymerase follow behind the exonuclease or act in the reverse direction (cf. Fig. 12.7)? Mutants of the three *uvr* genes all have the same phenotype and appear to be defective in the first step, the endonucleolytic excision of the damaged nucleotides.

Clark and Margulies (1965) isolated mutants of *E. coli* in which conjugation was normal but the ability of the DNA from the male cell to recombine with that of the female was defective. These mutants at the *rec* locus were highly sensitive to ultraviolet light, X-rays and various chemical mutagens. Both the recombination deficiency and the X-ray sensitivity were not shown by *uvr* mutants. At first it was thought that the *rec* mutants might be deficient in repair. It was found, however, that some repair is possible in these mutants which seem to have lost control of the change-over from DNA breakdown to replication, such that either less or more degradation than normal may occur. Howard-Flanders and Boyce estimated that instead of the average of 470 nucleotides normally released per pyrimidine dimer excised, the mutants *rec*-13 ('reckless') and *rec*-21 ('cautious') released 27,000 and 270, respectively. The nature of the defect in these mutants is not understood and it has not been conclusively established that the pathways of recombination and of dark repair share an enzyme, although this seems probable.

Rasmussen and Painter (1964) have found that tritiated thymidine is incorporated into the chromosomes of human cells in tissue culture following ultraviolet irradiation, and the effect is much enhanced if the thymine of the DNA has been partially replaced by 5-bromouracil. Control experiments without ultraviolet treatment showed no uptake of tritium, except in the fraction of cells undergoing normal DNA replication. It was inferred that a mechanism exists for the repair of ultraviolet damage to DNA, and that thymine is inserted into the DNA in the process.

From study of mutation caused by ultraviolet light in *Ustilago maydis*, Holliday (1962) came to the conclusion that both of the daughter cells arising from the first mitosis after irradiation were often mutant. In order to account for this, he suggested that the radiation modified a nucleotide (or nucleotides) in one chain, and that an enzymatically-controlled repair mechanism, in restoring normal hydrogen bonding, was liable to replace the complementary nucleotide in the other chain, so as to match the mutated one. The overall effect of ultraviolet light and 'repairing' enzyme would then be,

either to remove the effect of the ultraviolet light or to extend it to both chains. In other words, if ultraviolet light causes mispairing, that is, heterozygosity in the DNA, the repairing enzyme in restoring homozygosity may make the molecule homozygous mutant instead of homozygous normal. Holliday suggested that a similar process could be responsible for gene conversion if the DNA had become heterozygous at the mutant site through the pairing of complementary homologous chains.

I pointed out (Whitehouse, 1965) that on a hybrid DNA model of recombination, the 5 kinds of aberrant asci found by Kitani *et al.* (1962) with the grey-spored mutant of *Sordaria fimicola* (Fig. 16.1(*c*)–(*g*)) are what are expected if correction of heterozygosity in the DNA at the mutant site does not always happen, but may occur in either direction, that is, to homozygous normal (black) and to homozygous mutant (grey). If hybrid DNA occurred at the *g* site in both of the chromatids participating in crossing-over, and if there was no correction of the mispairing, asci with 4 black and 4 grey spores and with half of them not in pairs (Fig. 16.1(*c*)) would result. The 5:3 and 3:5 ratios would be the result of correction in one chromatid but not in the other, while the 6:2 and 2:6 ratios would be the consequence of correction in the same direction in both chromatids (see Fig. 16.7). Post-meiotic segregation, implying the persistence of mispairing through meiosis, might be due to failure of the postulated 'correcting' enzyme to reach its substrate during a critical period of time. One of the implications of the correction of heterozygosity in DNA as a source of recombination is that it can give rise to reciprocal as well as to non-reciprocal recombination. This is evident from Fig. 16.7, where correction in one direction in one chromatid and in the opposite direction in the other one will lead to normal reciprocal recombination, with asci containing 2 pairs of black and 2 pairs of grey spores.

Direct evidence that conversion is associated with the interior of a crossover, as the hybrid DNA models predict, was obtained by Rizet and Rossignol (1966) from study of *Ascobolus* asci segregating simultaneously for three closely-linked spore-colour mutants. Reciprocal recombination of the outer two was frequently associated with conversion at the intervening site and, moreover, the conversion involved the crossover chromatids.

The correction of mispairing in DNA at a mutant site would cause recombination simultaneously in two intervals of the chromosome, that is, between the mutant and neighbouring markers on both sides. Moreover, the hybrid DNA segments, where heterozygosity in the DNA arises, can occur only within crossovers and so there will be an association between crossing-over and the correction of mispairing. Whitehouse and Hastings (1965) assumed that there may be dissociation of the chains of the DNA molecules on one side of an opening-point, as illustrated in Fig. 16.6, or on both sides. The latter would give simultaneous crossovers in the same two chromatids in adjacent segments, with the result that outside markers would remain in parental combinations. This would account for the aberrant asci for the grey-spored mutant of *Sordaria fimicola* with parental combinations of outside markers (see § 16.2), and the similar results for genes in *Neurospora*

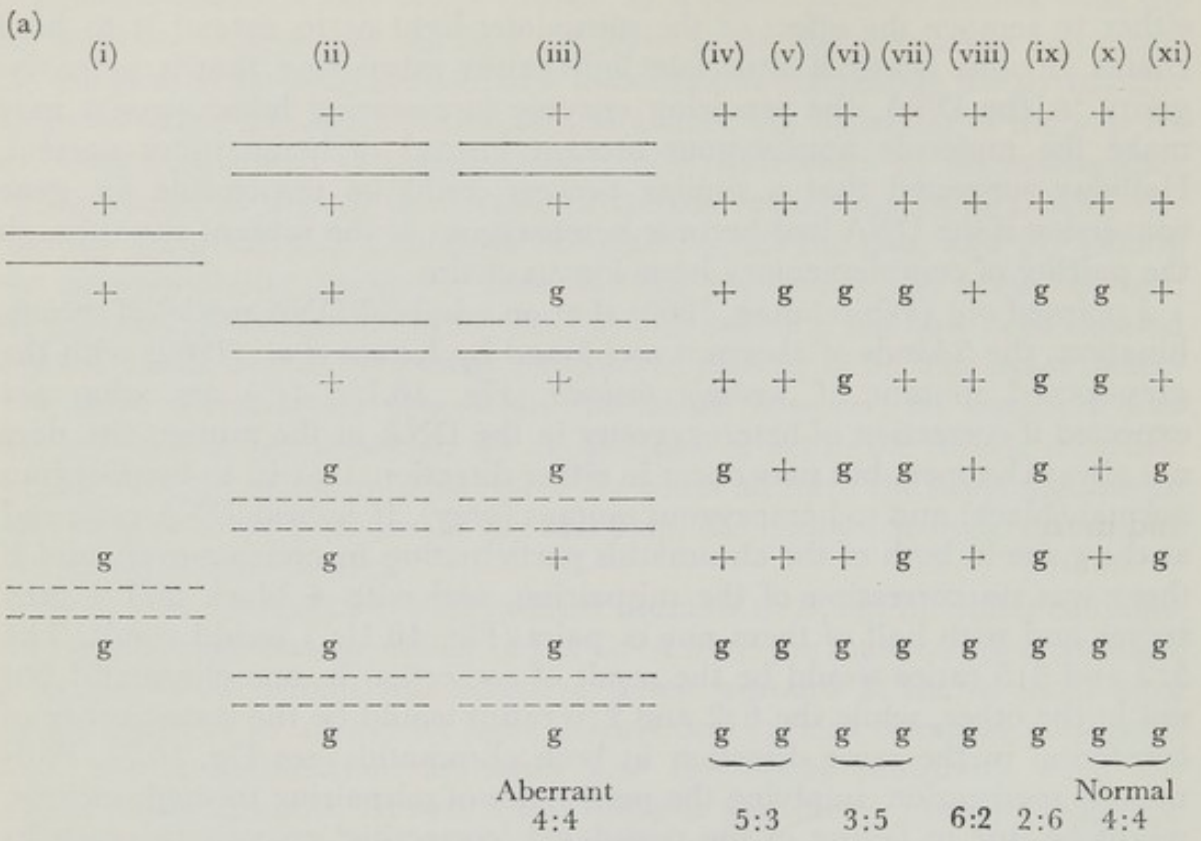


FIGURE 16.7 The diagram shows the origin of aberrant asci of *Sordaria fimicola* heterozygous for black (+) and grey (*g*) ascospore colour, according to hybrid DNA models of crossing-over. The pairs of horizontal lines represent DNA molecules (chromosomes or chromatids), the individual lines corresponding to nucleotide chains, and the full or broken lines distinguishing their parental origin.

The diagrams show (i) the parental chromosome-pair, (ii) the chromatids derived from them, (iii) the chromatids after a cross-over has occurred such that there is hybrid DNA in the region of *g* in two of the chromatids, and (iv)–(xi) the consequences of correction of the heterozygosity in one or both chromatids. In (ii)–(xi), the 8 nucleotide chains, each carrying either + or *g*, correspond to the 8 spores in the mature ascus. The numbers of spores of each colour in the asci are shown below the 8 symbols, with the number of black (+) spores given first. Asci of classes (iii), (v), (vii), (viii) and (ix) correspond to (*c*)–(*g*), respectively, in Fig. 16.1. The various permutations of spore sequence due to the presence or absence of a proximal cross-over, or to the chromosome orientation at the nuclear divisions in the ascus, are not shown.

and *Aspergillus*. Apart from these 'double crossovers', which are essentially single events in origin, there appears to be no evidence for the existence of negative interference between crossovers. It appears that recombination by correction of heterozygosity in DNA, together with the association between this correction and crossing-over, will account for negative interference. Pontecorvo (1958) pointed out that negative interference could be the result of the proximity to one another of the mutants necessary to detect it, if heterozygosis at one point along a chromosome favours recombination in its immediate vicinity, and in essence this is the explanation of negative interference provided by the hybrid DNA model.

As already mentioned in § 16.5, according to the model of recombination proposed by Holliday (1964), the primary breakage is in identical rather than in complementary chains in the two molecules, and there is no DNA synthesis. The secondary breakage, necessary to complete the crossover, may then occur in the same two chains, giving parental combinations for outside markers, or it may occur in the complementary chains, giving a crossover.

A hypothesis of recombination based on the correction of heterozygosity in DNA has to account for the fact that recombination frequencies between closely-linked mutants fit a linear map (cf. Fig. 16.2). The fit is often rather an approximate one, but nevertheless is well established. If recombination frequency depended merely on correction frequency, it would bear no relationship to the distance between sites. However, another factor to be considered is the frequency with which heterozygosity arises in the first place. If there is variability in the length of the hybrid DNA segments from a fixed origin, it is evident that mutant sites near the origin will occur in hybrid DNA more often than those further away. There will then be a gradient of decreasing conversion frequency with distance from the origin. It is evident that the unit of measurement of distance on a map of closely-linked mutants may not bear a constant relationship to the physical distance between sites. Where hybrid DNA is most frequent, the map may be relatively extended.

A further factor to be considered is the possibility that the heterozygosity itself interferes with the formation of hybrid DNA. This is particularly likely with deletions, to judge from the results obtained by Drake (1966) in experiments with virus *T*4 of *E. coli*. He studied the frequency with which *r*II mutants of known extent could be recovered from heterozygotes, and he found that the longer the addition or deletion the more often it failed to form a heteroduplex.

Howard-Flanders and Boyce (1966) obtained evidence that several hundred nucleotides are excised when ultraviolet-induced thymine dimers are removed from the DNA of *Escherichia coli*. If a similar length of chain were removed in the correction of mispairing in hybrid DNA, a second site of heterozygosity might be included in the excised segment. Both sites would then necessarily be corrected to the same parental genotype, and there would be no recombination between the two alleles. Two mutant sites close together in the DNA would be expected to show such linked correction more often than two sites further apart, and so the closer the mutants the lower their recombination frequency. This would explain the additivity of recombination frequencies of alleles. It would also explain why Lissouba and Rizet found that conversion did not occur at left-hand sites in series 46 of *Ascobolus* (Fig. 16.3): it is assumed that hybrid DNA forms from the right-hand end of the gene and when it extends to both mutant sites correction of mispairing of both occurs together and so does not cause recombinant asci. Only those asci in which hybrid DNA reached the first (right-hand) site but not the second would then be detected. Direct support for linked correction was obtained by Case and Giles (1964) who dissected *Neurospora* asci segregating for 3 alleles at the *pan*-2 locus and found several examples of

simultaneous conversion of all 3 to the same parent. Paszewski (1967) found that simultaneous conversion of two closely-linked spore colour mutants in *Ascobolus* was 7 times more frequent than would be expected if the conversion at the two sites had been independent.

Yanofsky *et al.* (1964) found that certain mutants affecting the *A* protein of tryptophan synthetase in *E. coli* showed consistently higher recombination frequencies than others, even though their sites were almost identical in position and within the same codon (see § 13.8). Moreover, the recombination behaviour was found to be associated with the amino-acid substitution which the mutation induced. Thus, mutants nos. 23, 27, 28, and 36, which led to the replacement of glycine by arginine at position no. 210 in the polypeptide, gave higher frequencies of recombination with allelic mutants than mutants nos. 46, 95, and 178, which caused the substitution of glutamic acid at the same position. It is inferred that the heterozygosity in the DNA in these two groups of mutants differ in their influence on hybrid DNA formation or on the correction mechanism.* In spite of this influence of individual mutants on recombination, it is also evident from the data of Yanofsky and associates that allelic recombination frequencies reflect the physical distance apart of mutant sites, as can be seen from Fig. 13.8.

Emerson (1966) reported that 571 *Ascobolus* asci showing aberrant segregation for mutant *w*-62 comprised 45·0% 6:2, 21·0% 2:6, 10·2% 5:3 and 23·8% 3:5 ratios of wild-type: mutant spores. (The frequency of the aberrant 4:4 class was not known.) This favouring of correction to wild-type in the even ratios ($6:2 > 2:6$) and to mutant in the odd ratios ($5:3 < 3:5$), which was not shown by the data of Kitani *et al.* for the *g* mutant in *Sordaria fimicola* (Fig. 16.1), implies that the pattern of correction is different in the two chromatids involved. Emerson suggested that this difference might be caused by a different kind of mispairing in each chromatid, such as Holliday's model predicts (for example, A-C and G-T with a transition). Another possibility is that the correction of mispairing is influenced by the presence of a break in a nucleotide-chain in the neighbourhood of the mispaired site. This idea is supported by the work on transformation in *Diplococcus pneumoniae* by Ephrussi-Taylor and Gray (1966), who proposed that correction of mispairing in the heteroduplex DNA took place with certain mutants which gave integration with low efficiency, and that this correction was strongly biassed in favour of excision of the donor chain. Such a bias implies enzymic recognition not only of a site of mispairing but also of some feature of the DNA which distinguishes donor and recipient, such as a free end to a nucleotide chain. Both the bacterial and fungal data could be explained if an enzyme complex, after recognizing a site of mispairing, 'scrutinizes' neighbouring regions of the DNA and, if a break is found, excises the broken chain. In biochemical terms this might mean that an endonuclease acts only if an exonuclease has failed to find its substrate. Selection might have favoured such a mechanism as it would prevent breaks near together in both chains.

* Drapeau, Brammar and Yanofsky (1968) found that 2 mutants which replace GAG (for glutamic acid) at codon 48 by the stop signal UAG increased recombination with close alleles up to 20-fold compared with 3 mutants which substitute another amino-acid at position 48.

Repair of mispaired bases in virus λ was demonstrated in an ingenious experiment by Hogness *et al.* (1967). Complementary nucleotide chains of λ differing by a mutation in gene *N* (Fig. 16.4) were annealed. Whether the mutation was in the heavy or the light chain (Fig. 14.6) made no difference to the activity of the wild-type allele, evidently because repair could transfer the wild-type base sequence to the chain that was transcribed. In confirmation of this conclusion, pre-irradiation of the host with a heavy dose of ultraviolet light evidently saturated the repair system, because the λ duplex having the *N* mutant in the heavy chain was then inactive. This experiment suggests that repair in either direction may occur equally often. In *E. coli*, as already mentioned, the dark repair system is apparently not specific to one kind of damage. These findings support the hypothesis that inequality in the frequencies of 6:2 and 2:6 ratios of aberrant asci, and likewise inequality of 5:3 and 3:5, is caused, at least in part, by the susceptibility of the correction mechanism to factors extrinsic to the mispairing itself (see also § 18.7).

Holliday (1967) isolated two recessive unlinked mutants (*uvs*-1, *uvs*-2) of *Ustilago maydis* which were sensitive to ultraviolet light and X-rays, and he found that they prevented ultraviolet-induced mitotic gene conversion. One of them (*uvs*-2) also blocked meiosis. The nature of the defect in these mutants is not known but, as with the *rec* mutants of *E. coli*, the results suggest that there may be steps in dark repair which are shared with recombination.

Zimmermann (1968) studied the phenotype resulting from mitotic gene conversion at the isoleucine-1 (*is*-1) locus in *Saccharomyces cerevisiae*. Conversion from mutant to wild-type was induced by N-methyl-N-nitrosourethane in a diploid heterozygous for two *is*-1 mutants *a* and *b*; this led to the production of the enzyme threonine dehydrase. Zimmermann found that 23 convertants were all identical with the wild-type for each of 9 properties of the enzyme that were examined. He concluded that conversion is an accurate process of information transfer, as predicted by hybrid DNA models.

In spite of this precision in conversion, the idea that crossing-over occurs with such molecular precision that no nucleotide is lost or gained must be qualified in view of the discovery by Magni and von Borstel (1962) that mutation at a number of nutritional loci in *Saccharomyces cerevisiae* is 6 to 20 times as frequent at meiosis as at mitosis and, moreover (Magni 1963), is often associated with crossing-over at or near the site of mutation. The strains used were initially homozygous at the loci where mutation occurred. Demerec (1962) observed a similar phenomenon in *Salmonella typhimurium*. Magni (1964) found that nutritional mutants in *Saccharomyces* thought to be due to base substitution (for example, because they were induced by base analogues) show at meiosis the same low rate of reverse mutation to wild-type as at mitosis. He inferred that the high mutation-rate at crossing-over is exclusively with mutations whose molecular basis is the addition or deletion of one or more nucleotides. Paszewski and Surzycki (1964) studied what appears to be the same phenomenon using a spore-colour mutant in *Ascobolus immersus*, and found that back-mutations affect 2 of the 6 spores in the ascus

and that, in the one ascus tested, the 6 mutant spores all behaved alike as regards further mutation. It is tentatively concluded that the phenomenon affects only one of the two chromatids taking part in crossing-over.

On hybrid DNA theories of crossing-over, it is possible that one or more nucleotides might be gained or lost if a nucleotide chain ended at the position of a run of identical bases, and if the tip of the chain initiated the contact with the complementary chain from the other molecule. Displaced pairing might then arise. For example, if the chain ended with a run of thymines, it might begin to associate with the complementary chain displaced by one nucleotide, thus:

```
---CCACCTTTGGAG
                G
                 T
                  TTT
     GGTGGAAACCTCCAAAATTACTGA---
```

This might lead to a distorted molecule:

```
                  T
---CCACCTTTGGAGG TTT
   GGTGGAAACCTCC AAAATTACTGA---
```

with the possibility of correction of the distortion by the addition of a nucleotide complementary to the unpaired one. Conversely, loss of a nucleotide could arise if the pairing was displaced in the other direction, and if the resulting distortion in the molecule were corrected by removal of the unpaired nucleotide. It is possible that the high spontaneous mutability at certain sites ('hot spots') in the *r*II region of virus *T*4 of *Escherichia coli* (see § 14.2) might be due to such displaced pairing. Streisinger, Okada *et al.* (1967) made the same suggestion to explain hot spots and obtained experimental evidence for it: the neighbourhood of mutant eJ42 in the lysozyme gene of *T*4 is a hot spot and is thought to have 5 adenines in a row (cf. Fig. 14.14(*a*)).

§ 16.7 Crossing-over as a gene-specific process

Whitehouse and Hastings (1965) concluded from study of all the relevant data that polarity in recombination was of general occurrence in Ascomycetes. From the pattern of this polarity it was inferred that the opening-points in the DNA where crossing-over is initiated are situated between the genes (or at their ends) and not within them. Most of the genes that have been studied show a reversal of polarity towards one end, as though hybrid DNA could be initiated at each end of every gene, but more extensively from one end than the other. I suggested (Whitehouse, 1966) that the DNA could be opened for recombination (that is, one chain broken and the pair dissociated) only at one specific end of each gene—the 'recombination-operator' end, but that the hybrid DNA from this starting-point might extend through this gene and into another. The predominant direction of polarity within a gene would then indicate at which end it was opened, while the weaker reversed polarity at the other end would be the overshoot of another gene.

This operator hypothesis is supported by the remarkable discoveries made by Catcheside and associates (Jessop and Catcheside, 1965; Catcheside, 1966; Jha, 1967) of dominant repressors of intragenic recombination in *Neurospora*. Each recombination-repressor seems to be specific for a particular structural gene (histidine-1 (*his*-1), amination (*am*), and *his*-3, respectively) and to be determined by a specific *rec* (recombination) gene which is not linked (or only loosely linked) to the gene on which it acts. The repressors do not eliminate intragenic recombination but reduce it 3- to 15-fold. Moreover, Jessop and Catcheside's data on polarity in *his*-1 can be interpreted as showing that the *his*-1 recombination-repressor abolishes the predominant direction of entry of hybrid DNA into *his*-1 (Whitehouse, 1966). This is what would be expected on the operator model.

As already pointed out, the process of episome integration in bacteria (§ 16.5) seems to resemble crossing-over in chromosomal organisms, not only in being a reciprocal exchange between two DNA duplexes but also in being controlled in a highly specific way. Moreover, there is a striking similarity between Jacob and Monod's specific repressors of gene expression in bacteria (§ 15.2) and Catcheside's specific repressors of gene recombination in a fungus. This raises the question of whether the transcription and the recombination of genes in chromosomal organisms are controlled by the same repressor. D. E. A. Catchside (1968) found, however, that the *am* recombination-repressor did not repress production of the glutamate dehydrogenase specified by *am*.

Another problem is how regulatory genes undergo recombination. This is unlikely to be by crossing-over if this process is regulated by such genes. There is a possibility, both from the work of McClintock with controlling elements in *Zea mays* (see Chapter 18) and that of Ellingboe (1965) with incompatibility factors in *Schizophyllum commune*, that recombination of regulatory genes may be by episomes.

If crossing-over can be initiated only at a gene end and not within it, this represents a step back towards the old bead hypothesis of the gene, but with a significant difference. The old idea (§ 9.9) was that crossing-over was confined to the links between genes, but it is now suggested that crossing-over is merely initiated at the end of a gene and then the molecular processes involved extend into it. Moreover, there is a secondary process of recombination, namely, the correction of heterozygosity, which occurs only when a heterozygous gene lies within a crossover, and is responsible for the phenomenon of conversion.

The possibility is discussed in § 18.9 that the initial step in crossing-over is not breakage of nucleotide-chains but failure to join them at replicon junctions after the premeiotic DNA replication.

17. *The theory of the single-stranded chromosome*

§ 17.1 Single-stranded *versus* multistranded chromosomes

Ever since the establishment of the DNA theory of the molecular basis of heredity (Chapter 11), there has been controversy over the question of whether the germ-line chromosome is single-stranded or multistranded. According to the single-strand hypothesis, the chromosome axis consists essentially of only a single DNA duplex (or a number joined end-to-end), while the multistrand hypothesis supposes that the chromosome is built like a cable with the genetic information repeated in two or more DNA molecules lying parallel to one another. The single-strand model is favoured by most geneticists because of the difficulty otherwise of explaining mutation and recombination. The multistranded structure has been favoured by many cytologists. The evidence relating to this controversy is reviewed in this chapter.

§ 17.2 Light microscopy of chromosomes

Claims that telophase chromosomes are multistranded have been made on many occasions on the basis of their appearance under the light microscope. References to such reports are given by Bajer (1965) who has found from phase-contrast time-lapse photography of mitosis in living endosperm cells of *Haemanthus katherinae* that the chromosomes are sometimes clearly double at anaphase and telophase. Trosko and Wolff (1965) observed four strands in the chromosomes of *Vicia faba* root-tip cells after trypsin digestion and concluded that the chromosomes are multistranded. Maguire (1966) observed double-stranded chromosomes in *Zea mays*. It is manifest, however, that light microscope observations, with a resolving-power of 0·2–0·3 μ (Bajer 1965), cannot distinguish between a multistranded condition and one very long thread less than 0·2 μ wide and folded into aggregates. Taylor (1966), in referring to Trosko and Wolff's inference of multistrandedness, stated that "such a conclusion is obviously unjustified".

§ 17.3 Electron microscopy of chromosomes

From study of electron micrographs of chromosomes from a number of different plants and animals at various stages of mitosis and meiosis, Ris

(1957) came to the conclusion that a chromosome is multistranded in the sense that it is built like a cable with numerous identical sub-units. Kaufmann and McDonald (1957) reached similar conclusions. However, the possibility that the chromosome is single-stranded does not appear to be excluded by these observations because the multiple strands observed with the electron microscope might represent a single strand folded back and forth upon itself a number of times.

Gall (1966) used the technique of spreading material at an air-water interface, first developed by Kleinschmidt *et al.* (1962) (see § 11.3), and applied it to chromosomes. By electron microscopy after such treatment he observed fibres of diameter 25–30 mμ in chromosomes from *Triturus* erythrocytes and human cells in culture. He saw no evidence for a multistranded condition within these fibres and from their size and their ability to stretch he believed they contained one coiled or folded nucleohistone molecule. Wolfe (1965) and DuPraw (1965) reached a similar conclusion using Gall's technique applied both to interphase nuclei and to metaphase chromosomes of *Triturus*, *Bos* and *Apis mellifica*. The problem of the number of strands per chromosome then resolves into how many of these fibres make up a chromosome. In this connection, Wolfe and Hewitt (1966) made a significant observation. They found that the number of 25 mμ fibres in the chromosomes of the hemipterous insect *Oncopeltus fasciatus* increases during prophase of meiosis from 15–40 at zygotene to 25–50 at pachytene and to 30–65 at diakinesis; all counts refer to bivalents, that is, four chromatids. Since it is well known that chromosomes show thickening and contraction in length during prophase, Wolfe and Hewitt concluded that the increase in the number of fibres seen under the electron microscope is caused by parallel folding. They argue from this that it is quite possible that the multistranded appearance seen at leptotene and zygotene is also caused by such folding.

Miller (1964*b*) reported briefly that lampbrush chromosomes (§ 17.6) of *Triturus viridescens*, after digestion with proteases and ribonuclease, revealed under the electron microscope a thread 5 mμ wide between chromomeres and 3 mμ wide in the lateral loops. The interchromomeric region in *Triturus* lampbrush chromosomes is thought to correspond to two chromatids in close association, and the lateral loops to one chromatid. Since the DNA molecule is 2 mμ wide, Miller's observations, if confirmed, would establish that the chromosome is single-stranded as regards its DNA.

§ 17.4 Tritiated thymidine labelling

If chromosomes replicate once in the presence of tritiated thymidine and then once in an unlabelled solution, each pair of sister chromatids is usually labelled in one chromatid and not in the other, though sister-chromatid exchanges may occur (§ 12.2). Corresponding segments of both chromatids, or even the whole of them, however, are sometimes found to be labelled at this second mitosis (La Cour and Pelc, 1958; Peacock, 1963). This isolabelling, as it has been called, is usually interpreted as evidence that the

chromosome is multistranded: for instance, as though the diagrams in Fig. 12.2(*b*) represented single chromatids with 4 sub-units.

The iso-labelling can be explained, however, with a single-stranded chromosome. Peacock illustrates (his Fig. 6) an example of an intercalary iso-labelled segment separating singly-labelled segments that have undergone an exchange. In order to reconcile cytological observations with genetic data on recombination, I have suggested (Whitehouse, 1967) that the chromomeres are detached before crossing-over occurs at meiosis (see Chapter 18). Iso-labelling would be accounted for if chromomeres, detached for sister-strand exchange, were sometimes restored to the sister chromatid.

As mentioned in § 12.2, strong support for the single-strand hypothesis of the structure of the chromosome is given by Herreros and Giannelli's (1967) confirmation of the 2:1 ratio of single:twin exchanges, implying that the two longitudinally-arranged sub-units of the chromosome, which replicate semi-conservatively, are structurally different from one another.

§ 17.5 X-ray-induced aberrations

It has been known for some time that doses of X-rays applied to cells in G1 (the first gap) between telophase and the period of DNA synthesis (S period) give rise in general to chromosome-type aberrations at the following metaphase, whereas irradiation in G2 (after the S period) causes chromatid-type aberrations such as those discussed in § 9.8. These results are expected if the division of the chromosome into two chromatids coincides with the period of DNA synthesis because chromosome-type aberrations have both chromatids involved in similar structural changes, as though the aberration occurred before chromosome duplication. Evans and Savage (1963) and Wolff and Luippold (1964), however, independently discovered, by the use of ^{3}H-thymidine labelling in conjunction with X-irradiation, that the chromosomes of *Vicia faba* become double to X-rays in late G1 about one hour at room temperature before DNA synthesis begins. This discovery has been used as an argument for the multistrandedness of the chromosome. A single-strand model, however, does not seem to be excluded when the probable steps in the formation of aberrations are considered, using such a model.

Damage to chromosomes by X-rays is likely to involve breakage of single nucleotide-chains, either as a direct effect, or indirectly through the enzymic excision of damaged bases (§ 16.6). The formation of structural aberrations is probably caused by chance encounters between unpaired chains of non-homologous origin exposed in this way. Annealing would occur if there were segments in antiparallel juxtaposition with a sufficient length of a mutually complementary sequence of nucleotides. Subsequently, on the basis of what is known from *Escherichia coli*, repair of DNA would be expected to occur, and to require the successive action of an exonuclease to erode one chain in order to enlarge a gap, a polymerase to fill the gap, and a ligase to seal it. An endonuclease would also seem to be needed at some stage of repair in order to cut one chain where Y-shaped configurations occurred; depending on which chain was cut, a potential aberration would either be fixed or

removed. Evans (1967) proposed a similar 'misrepair' model for the formation of aberrations. He suggested that in *Vicia faba* root-tips grown at room temperature the repair of DNA following X-ray damage occurred 5 to 6 hours after irradiation, because he found a fractionated X-ray dose with this time interval caused as many aberrations as a single dose of the same total size given at the earlier time. He suggested that during repair the chromosomes were temporarily more vulnerable to the formation of aberrations. In molecular terms, the single chains exposed through breakage by the first half-dose are exposed again during repair, with the possibility of chance contact with chains exposed through breakage by the second half-dose.

If aberrations are not fully defined until DNA repair occurs, it is possible that the single-strandedness or double-strandedness of chromosomes to X-rays, that is, whether they form chromosome-type or chromatid-type aberrations, is a reflection of their condition, not at the time of irradiation, but at the time of the subsequent repair of the DNA. It seems significant that the time of transition from singleness to doubleness to X-rays (late G1) corresponds to a time for DNA repair (allowing a 5–6 hour interval) in the middle of the S period, since this lasts about 8 hours at room temperature. On this interpretation, therefore, the X-ray aberration data are in keeping with a single-strand model for the chromosome.

§ 17.6 Lampbrush chromosomes

The chromosomes at diplotene of meiosis in female newts show a remarkable feature which has earned them the name of lampbrush chromosomes (review: Callan, 1963; see § 8.13 and Plates 6 and 7). The chromosomes at this stage are very long (up to 800 μ) and have hundreds of loops projecting laterally for 10–15 μ or more, giving a fuzzy or lampbrush appearance. The loops arise in pairs from chromomeres (Fig. 17.1(*d*)), and are thought to be associated with the functioning of some of the genes, since RNA and protein synthesis have been demonstrated autoradiographically as occurring alongside them (see § 18.7). This synthesis is no doubt related to the considerable growth of the oocytes which occurs during the period of at least 200 days that the nucleus remains at the diplotene stage. The nucleus itself increases greatly in size during this period. Callan found that when a lampbrush chromosome is stretched, the chromomeres break transversely and a pair of lateral loops spans the break (Fig. 17.1(*e*) and Pl. 7(*b*) and (*c*)). He inferred that the loops have an axis which is continuous with the fine thread which joins the chromomeres, and forms the main axis of the chromosome. From electron micrographs of lampbrush chromosomes after pepsin digestion, Gall (1956) confirmed that there is an axis to the lateral loops, and moreover showed that it was resistant to the enzyme. His interpretation of the structure of a lampbrush chromosome is shown in Fig. 17.1(*f*). Callan and Macgregor (1958) transferred unfixed *Triturus* lampbrush chromosomes to physiological saline, and studied the effect of enzymes on them. They found that pepsin and trypsin, which split peptide linkages near free acidic and basic groups respectively, and ribonuclease, which breaks down RNA, did not destroy the linear integrity of the chromosomes, whereas deoxyribonuclease (DNase)

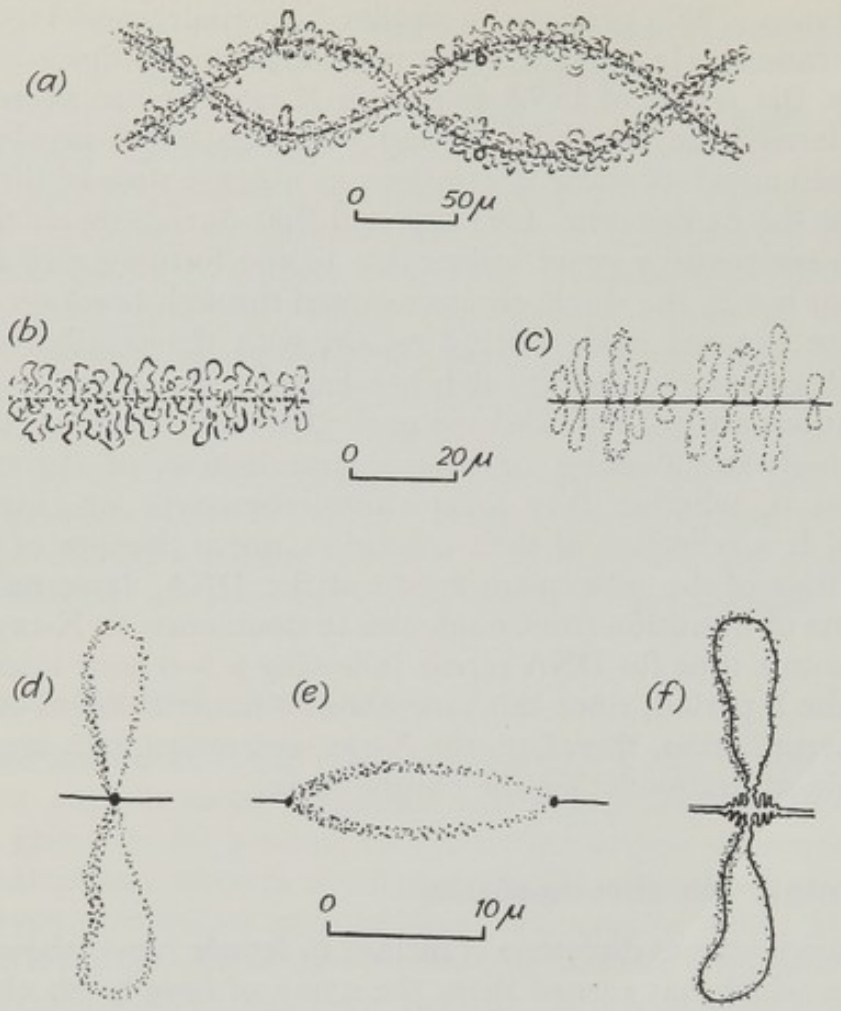


FIGURE 17.1 Drawings to illustrate the appearance of the chromosomes at the diplotene stage of meiosis in oocytes of *Triturus*.
(*a*) One chromosome-pair with three chiasmata.
(*b*) Part of one chromosome, to show the lampbrush appearance in more detail.
(*c*) Diagrammatic representation of (*b*) to illustrate how the loops arise in pairs from the chromomeres.
(*d*) One such pair of loops in more detail.
(*e*) The appearance of such a loop-pair after stretching (from Callan).
(*f*) The structure of a loop-pair, according to Gall (1956).

which breaks down DNA, had a dramatically different action: the lateral loops and the thread joining the chromomeres rapidly disintegrated. They inferred that DNA runs throughout the length of the chromosome, including the axis of the lateral loops. Dutt and Kaufmann (1959) observed no disruption of meiotic chromosomes in males of the grasshoppers *Trimerotropis maritima* and *Melanoplus femur-rubrum* when unfixed material was treated with DNase, but Macgregor and Callan (1962) attribute this to the relatively compact condition of the chromosomes at the time of treatment, compared with the remarkably extended diplotene chromosomes in female *Triturus*.

Macgregor and Callan have found that much contracted parts of these amphibian chromosomes, such as the chromomeres, also fail to show breakage by DNase.

Gall (1963) studied the kinetics of the DNase breakage of *Triturus* lampbrush chromosomes. Unfixed chromosomes were photographed at intervals over a period of one hour after placing in the enzyme solution. From the photographs the number of breaks was counted and plotted against the time. Breaks in the lateral loops were scored separately from breaks in the main axis. It was assumed that the chromosome consists of n longitudinal sub-units, that each is attacked independently and with equal probability by the enzyme, and that all of them have to be digested at approximately corresponding positions in order to cause a break in the chromosome. The number of breaks will then be directly proportional to the time if there is one sub-unit, proportional to the square of the time if there are two sub-units, to the time cubed if there are three, and so on. Gall obtained the values $n = 2.6 \pm 0.2$ for the loops, and $n = 4.8 \pm 0.4$ for the main axis. He considered the true sub-unit numbers to be 2 and 4, respectively, because delay in penetration of the enzyme through the nuclear sap, and delay in observing a break after it had occurred, would both tend to raise the observed values of n above the true one. That there should be twice as many sub-units in the main axis as in the loops is in keeping with his earlier interpretation of the structure of lampbrush chromosomes (Fig. 17.1(f)), according to which the loops are parts of the axis of individual chromatids, while the main axis consists of a pair of sister chromatids in close association with one another. From *in vitro* studies, Thomas (1956) found evidence that DNase cleaves the phospho-ester links in the two nucleotide chains of DNA independently and at random, and that breakage of the molecule occurs only when the two chains are severed less than about 3 nucleotide-pairs apart. Gall inferred that the two sub-units of the chromatid were the two nucleotide chains, and that the chromatid contains either one very long DNA molecule or a series of DNA molecules connected end-to-end. Gall's results thus provide strong support for the single-strand hypothesis.

Lampbrush loops are not confined to the chromosomes of amphibian oocytes. They have been seen at diplotene in the oocytes of a wide range of animals (see Callan and Lloyd, 1960) and in the spermatocytes of insects: at least 4 loop-pairs were observed by Hess and Meyer (1963) in the Y-chromosome of *Drosophila hydei* and numerous loops were seen by Henderson (1964) in the autosomes of orthopteran insects (see Plate 3(a) and (b)). Lampbrush loops were also observed at diplotene in *Allium cernuum* by Grun (1958), who gave reasons for believing that these loops are of general occurrence at this stage. Henderson considered it likely that all prophase chromosomes have the lampbrush organization.

§ 17.7 Induced subchromatid exchanges

Exchanges which appear to be between sub-units of chromatids were first clearly described by Swanson (1947). These aberrations were seen at

anaphase in the pollen-tube mitosis of *Tradescantia*, following X-ray treatment at the preceding prophase. The anaphase bridges with two side arms caused by these subchromatid exchanges have since been observed in many organisms, and at meiosis as well as mitosis. If they were due to subchromatid exchange, as most observers have inferred, this would imply that the chromatid was at least two-stranded. It is possible, however, that these exchanges, when induced by irradiation or chemicals, occur between lampbrush loops, either between those of sister chromatids or of non-sisters, or between non-homologous loops. Assuming that the axis of the loop is continuous with the chromatid axis (Fig. 17.1(*f*)) such an exchange would really be between whole chromatids. The subchromatid appearance would be due to a failure of transverse breakage of the chromomeres whose loops underwent exchange; such breakage would not be expected in contracted chromosomes. Subchromatid exchanges are normally induced only at mitotic prophase or late prophase I of meiosis, which are the stages when chromosomes are known or believed to have the lampbrush configuration (§ 17.6).

Irradiation or chemical treatment in interphase does not produce exchanges of subchromatid appearance at the next mitosis. In interphase the chromosome or chromatid is not aggregated into chromomeres, and consequently, with an exchange at positions corresponding to lampbrush loops, the double association by loop axis and by chromomere would not be expected. An interphase exchange will thus not be expected to give rise at mitosis to the subchromatid appearance characteristic of exchanges induced at a time when the chromosomes are strongly contracted. Taylor (1958) has obtained direct experimental evidence by autoradiography with ^{3}H-thymidine that subchromatid exchanges are not induced in the S or G2 periods of interphase.

Induced subchromatid exchanges, if between half-chromatids, would be expected to give rise to chromatid-type aberrations at the next mitosis. On the other hand, if subchromatid exchanges are really between lampbrush loops and hence whole chromatids, they would be expected to give rise at the next mitosis to chromosome-type aberrations. One of the characteristic features of induced subchromatid exchanges is the rarity of chromatid aberrations at the next division, in agreement with the present hypothesis. Moreover, Östergren and Wakonig (1954) and Kihlman and Hartley (1967) found numerous chromosome-type aberrations in *Allium cepa* and *Vicia faba* root-tips at the mitosis following that at which subchromatid exchanges had been induced.

§ 17.8 DNA content of nuclei

One of the main arguments for the multistranded chromosome has been based on the variation in DNA content of the chromosomes of related organisms. Both animals and plants show this variation to an extraordinary degree. Mirsky and Ris (1951) measured the DNA content of erythrocytes of various animals and found great variation. Expressed in picograms (see § 11.4) per diploid cell, the values for 31 teleost fish ranged from 0·94 to 3·49, with figures of 5·46 and 6·67 for two elasmobranch fish (*Carcharias* spp.)

and 100 for a dipnoan fish (*Protopterus* sp.). Two anuran amphibians had values of 7·33 and 15·0, and two urodelan species 48·4 and 168. McLeish and Sunderland (1961), using root-tip cells, found similar variation in flowering plants: 5·5 to 60·5 in 4 dicotyledons and 15·5 to 107·7 in 5 monocotyledons; these figures do not include polyploid species. Rothfels *et al.* (1966) obtained figures ranging from 1·3 to 52·5 for 22 diploid members of the Ranunculaceae. It is evident that within some groups of organisms such as fish, amphibians and flowering plants, DNA contents per cell can vary 20- to 100-fold.

Associated with the variation in the amount of DNA is a variation in chromosome size: Rothfels *et al.* found that in the Ranunculaceae the total length of the metaphase chromosomes increased approximately as the square root of the DNA content, ranging from 20 to 220 μ in the species in which the DNA was measured.

The great variation in DNA content is found even between species of the same genus. Schrader and Hughes-Schrader (1956) measured the density of Feulgen staining at prophase I of meiosis in males of two species of hemipterous insect of the genus *Thyanta* and found in this way that one had 25% more DNA than the other, although they had the same chromosome number. Rothfels *et al.* found a 3·5-fold range of variation in diploid species of *Anemone*, and Martin and Shanks (1966) and Rees *et al.* (1966) a sixfold range in diploid *Vicia* spp. Rees *et al.* also found a 2·3-fold range in *Lathyrus* species with 14 chromosomes (2*n*). Callan (1967) quoted the finding of a threefold variation within the crustacean genus *Gammarus* and 11-fold in the flatworm genus *Mesostoma*, again between species with the same chromosome number.

Moreover, the specific differences in DNA content are an intrinsic property of the particular chromosomes. This was shown by Ullerich (1966). He found that the toad *Bufo bufo* had 40% more DNA per cell than *B. viridis*. Both species have the same chromosome number: 2n = 22. He measured the relative DNA content of individual chromosomes determined from the density of Feulgen stain. He found that *bufo* chromosomes nos. 5, 7 and 8 in order of decreasing size had about 53% more DNA than their homologues in *viridis*, and the other 8 had about 36% more. The same differences were found in a hybrid of the two species.

Recent studies with the giant salivary gland chromosomes of dipteran larvae (§ 9.7) have solved the problem of the source of these large variations in DNA content. This was an unexpected development because these chromosomes are clearly abnormal, and unquestionably multistranded (*polytene*), and might not have been expected to provide information relevant to germ-line chromosomes. The correspondence, however, between chromomeres and the transverse bands of these chromosomes has been established. Beermann and Pelling (1965) used ^{3}H-thymidine to label the DNA of fertilized eggs of *Chironomus tentans*. Autoradiographic examination of the chromosomes of the salivary glands of the larvae showed that some had a radioactive thread resembling a leptotene chromosome extending the length of an otherwise unlabelled giant chromosome (see Pelling, 1966).

Keyl (1965*a*, *b*) studied two subspecies, *thummi* and *piger*, of *Chironomus thummi* and found that the type subspecies had 27% more DNA per cell than *piger*. In genera such as *Drosophila* and *Chironomus* it has been possible to establish evolutionary relationships between species from study of the salivary gland chromosomes because structural changes in these chromosomes can be recognized by identifying the sequence of transverse bands (§ 9.7). From comparison of the salivary gland chromosomes of the two subspecies with those of other species of *Chironomus*, Keyl concluded that *piger* is the nearer of the two to the ancestral stock. He inferred that an increase in the amount of DNA in the chromosomes had occurred in the evolution of subspecies *thummi*, since *piger* has the lower value.

TABLE 17.1 Data of Keyl (1965*b*) for the DNA content of particular transverse bands of the salivary gland chromosomes of *Chironomus thummi* subspecies *thummi* relative to subspecies *piger*.

Chromosome	Band	DNA Content					Chromosome	Band	DNA Content			
IR	c3/3		×2				IIR	b5/28	×1	×2	×4	
	4	×1						c1/7	×1	×2		
	5	×1						8	×1	×2		
	6	×1	×2					9	×1	×2		
	c4/1			×4				11		×2	×4	
IL	d3/8	×1	×2					c2/1	×1			
	e1/2	×1	×2					2	×1			
	3	×1						3	×1	×2		
	4		×2					4		×2		
	6	×1						11		×2		
	7		×2					12			× 4	
	e2/3–5	×1					IIL	c4/3		×2		
III	b1/13				×8	×16		6			×4	
	b3/11		×2	×4	×8	×16		7		×2	×4	
	17		×2					12		×2		

Keyl measured the relative DNA content of individual transverse bands by microspectrophotometry after Feulgen staining. When he compared the DNA contents of corresponding bands in the hybrid of the two subspecies he made a remarkable and quite unexpected discovery. The DNA content of each band of *thummi*, regardless of the absolute amount, always differed from that of its counterpart in *piger* by a power of 2: either 1, 2, 4, 8 or 16 times as much DNA in the one as the other. Different individuals of *thummi*, particularly when derived from a different population, sometimes differed in the DNA content of certain bands (see Table 17.1). Moreover, this variation was not caused by more rounds of replication in some bands than others during the development of the salivary gland chromosomes, because the total DNA content of the *thummi* cell was 1·27 times that of *piger* whether measured in the salivary glands or the spermatocytes. Evidently the germ-line chromosomes of subspecies *thummi* also showed the geometric differences from *piger* in the DNA content of individual chromomeres collectively responsible for the 1·27:1 ratio.

The source of the variation in DNA content of related organisms was thus revealed as a successive doubling of the amount of DNA in individual chromomeres. Moreover, Keyl had reason to believe that this doubling was in the length and not the number of strands within each chromomere because Keyl and Pelling (1963) had found by ^{3}H-thymidine autoradiography that the larger bands take longer to replicate, as though they contained a greater length of DNA. Further aspects of Keyl's discovery, which is clearly of fundamental importance for an understanding of chromosome organization, are discussed in Chapter 18.

§ 17.9 Conclusions about chromosome strands

From the discussion of the cytological evidence for the multistranded chromosome in §§ 17.2–8, it is evident that none of the data provides good evidence for multistrandedness, and in no instance is the single-strand alternative eliminated. Moreover, the results obtained by Taylor, Herreros and Giannelli from ^{3}H-thymidine labelling, and by Callan, Gall and Miller from study of lampbrush chromosomes, provide strong arguments in favour of single-strandedness.

Sueoka, Chiang and Kates (1967) investigated the DNA content of the cells of an abnormal strain of *Chlamydomonas reinhardi* which produced 8 zoospores per zygospore instead of the normal 4; they also studied the DNA replication using the heavy isotope of nitrogen (^{15}N) as a density label. They found that the vegetative cells and gametes of the 8-spored strain contained 0·123 pg of DNA and the zygospores 0·246 pg. The gametes were evidently in the G2 stage because no further round of replication occurred before meiosis began. Sueoka *et al.* discovered that the DNA replicated 8 hours (at 25°) after zygospore germination had been initiated by exposing the spores to light. This time corresponds to prophase I of meiosis but was probably after the time of crossing-over because the zygospores of the 8-spored strain produce 4 pairs of zoospores in which the two spores of each pair are genetically identical. No further DNA replication took place before the postmeiotic mitosis had been completed and the 8 zoospores released, some 12 hours after the initiation of germination. Evidently the DNA synthesis at 8 hours was the pre-mitotic DNA replication, and the zoospores when first formed were in G1. The telescoping of the normal alternation of DNA replication and nuclear division may be related to the precocity of the postmeiotic mitosis, which occurs within a few minutes of the completion of meiosis. The results do not contradict the single-strand theory of the chromosome.

It has been apparent for many years that mutation and recombination are phenomena that are normally shown by chromatids rather than by sub-units within the chromatid. For recombination, this is manifest from the results of tetrad analysis (cf. § 8.6), although postmeiotic segregation (see § 16.2) constitutes an exception. With the acceptance of the DNA theory of the molecular basis of heredity, it appeared most improbable that several DNA molecules carried the same genetic information within one chromatid. If a mutation can represent a change in only one nucleotide-pair, and if, as would

appear, recombination normally occurs with such precision that no nucleotide is lost, added or changed*, the simultaneous occurrence of any particular gene in more than one DNA molecule in a chromatid is most unlikely. This argument applies only to the germ-line, and it is evident that in certain differentiated cells the chromosomes are multistranded (see § 9.7 and § 17.8). As indicated in Chapter 16, data on recombination are in agreement with hybrid DNA theories of crossing-over based on the assumption that a chromatid can be looked upon as one DNA molecule (or as a number joined end-to-end). The recombination data contradict hypotheses of chromatid structure which postulate a multistranded condition, since these would require exceedingly elaborate and implausible models to account for the data on conversion, postmeiotic segregation and crossing-over. It is evident that the genetic data confirm the cytological observations of Taylor with *Vicia* and *Bellevalia* root-tips, and of Callan and Gall with *Triturus* oocytes, and establish that a chromatid is a single-stranded structure in the sense that each gene is represented in only one DNA molecule.

The simplest hypothesis of chromosome structure which fits the data is that the whole of the DNA of a chromosome is contained in one giant DNA molecule. Gall (1956) has estimated that the 12 chromosomes in the haploid set of *Rana pipiens* contain an average of 20 cm of DNA if it were straightened out, and the 11 chromosomes of *Triturus viridescens* an average of 90 cm. It is evident from labelling experiments that the chromosome replicates in discrete segments in a controlled sequence, and not progressively from one end (§ 12.7). This means that during replication a number of separate DNA molecules will exist, but as indicated in § 12.7, the simplest explanation of the data is to suppose that when replication is completed these are joined end-to-end into a single giant DNA molecule.

If the central axis of a chromosome is a single DNA molecule, how does the protein fit into its construction? The unequal spacing of the two nucleotide chains about the DNA helix axis leaves two spiral grooves. Atoms nos. 2 and 3 of the purines and pyrimidines lie at the bottom of the smaller groove, and atoms nos. 5 and 6 at the bottom of the deep groove (cf. Fig. 11.5). Watson and Crick (1953*b*) pointed out that the molecular dimensions of DNA were such that a fully extended polypeptide chain could be wound round the same helix axis as the nucleotide chains. Feughelman *et al.* (1955) confirmed this for nucleoprotamine, the nucleoprotein found in the sperm of some animals. Protamine appears to consist of one polypeptide chain in which about two-thirds of the amino-acid residues are arginine. Feughelman and associates showed from X-ray crystallographic evidence that the polypeptide chain lies in the smaller groove. The spacing is such that the basic end-groups of the arginine side-chains of the polypeptide can combine with the phosphate groups of the DNA, while non-basic amino-acid residues probably occur in pairs at folds in the polypeptide chain.

Wilkins (1957) tentatively suggested that in nucleohistone, which appears to occur universally in chromosomes except in the sperm of some animals, there might be a polypeptide chain in both grooves of the DNA. However,

* The rare occurrence of mutation in association with recombination is discussed in § 16.6.

the *X*-ray diagrams were much less clear than for nucleoprotamine, and the structure of nucleohistone remains uncertain. Pardon, Wilkins and Richards (1967), from X-ray diffraction studies, obtained strong evidence that nucleohistone is folded in some regular way with the DNA inclined to the fibre axis. They suggested that a nucleohistone thread of 3 mμ diameter might be coiled to give a helix of pitch 12 mμ and of diameter 10 mμ measured from the axial centre of the thread, that is, 13 mμ outside diameter. The discrepancy between this fibre thickness inferred from X-ray diffraction and that observed by DuPraw, Wolfe and Gall in electron micrographs (20–25 mμ) is unresolved.

Kellenberger (1961) has discussed the process of condensation of DNA in the formation of the head in bacterial viruses. The DNA of the head occupies only about one-fifteenth of its previous volume, through removal of water molecules. The condensation process does not occur in the presence of chloramphenicol, which is an inhibitor of protein synthesis. When virus *T*2 of *Escherichia coli* is ruptured by osmotic shock, small quantities of proteins and polyamines are released, in addition to the DNA. It is inferred that protein plays a part in the condensation process. It appears that the polyamines may have a similar function, since Kaiser, Tabor and Tabor (1963) found that the polyamine spermine protects DNA from breakage by hydrodynamic shear. Spermine has the chemical formula:

$$NH_2 . (CH_2)_3 . NH . (CH_2)_4 . NH . (CH_2)_3 . NH_2$$

These authors found that if an aqueous suspension of DNA obtained from virus λ of *E. coli* is stirred, genes placed well apart on the linkage map are inherited independently, indicating that the DNA has been broken. The marker genes were found often to stay together in the progeny if spermine was added before stirring the DNA. Only a minute quantity of spermine was required. It was suggested that the spermine might make the DNA more resistant to shear, either by alignment of the aliphatic chains along the helix, or by pulling together phosphate groups from different parts of the molecule to give a more compact configuration.

It is possible that the basic proteins (protamine or histones) in chromosomes have a similar function to the polyamines and protein in the heads of bacterial viruses. The protein part of the chromosome may be responsible for its characteristic morphological features, such as chromomeres and spiral coiling, since bacterial chromonemes, which lack these features, also lack protein in association with their DNA. That protamine should occur in association with the chromosomes in the sperm of some animals, instead of the histones found in other nuclei, may be related to the smaller size of the protamine molecule allowing the chromosomes to become more compact. For sperm, small size means greater mobility and hence has a selective advantage.

The histones of the chromosomes are known to show species specificity, and this suggests that their function is not merely to protect the DNA from breakage, and to allow it to fold into condensed configurations. It is possible that they are concerned in the control of gene action in cell-differentiation. This is discussed in § 18.9.

18. *The theory of the chromomere as the basic unit of the chromosome*

§ 18.1 Introduction

The chromomere is a characteristic feature of chromosomes, particularly in early prophase I of meiosis and, as transverse bands, in the giant salivary gland chromosomes of Diptera (Fig. 9.1). Suggestions such as that of Belling (1928*b*) (see § 9.9) that chromomeres correspond to genes have received little support because the chromomere was much too large to correspond to 1000 or 1500 nucleotide-pairs of DNA and associated proteins. Recent work, however, suggests that the chromomere may, nevertheless, be the basic unit of chromosome organization. The control of gene action is discussed in this chapter with particular reference to the chromomere. One way in which information about this control has been obtained has been from study of the causes of instability in gene expression, such as give rise to mottled colouring or other kinds of variegation.

§ 18.2 Unstable phenotypes

Emerson (1914) was the first to show that a variable or unstable phenotype may be caused by mutations occurring with abnormally high frequency. He studied the variegated pigmentation of the pericarp found in some strains of *Zea mays* and established that the coloured stripes were due to mutation to a dominant allele of a gene affecting the pigment, for he found that the coloured condition was sometimes inherited. This was evidently when the mutation had occurred in the germ-line.

An outstanding example of a variegated phenotype resulting from mutation is due to the work of Rhoades (1936, 1938), also with *Zea*. The production of red or purple anthocyanin pigment in the aleurone layer of the endosperm of the maize grain requires the simultaneous presence of four dominant genes called A_1, A_2, C, and R, and situated in chromosomes nos. 3, 5, 9, and 10, respectively. After self-pollination of a maize plant homozygous for all these dominant factors, Rhoades (1936) found an ear with grains in the proportion of 12 with coloured aleurone: 3 dotted: 1 with colourless aleurone, instead of all the grains coloured as expected. The dotted aleurone character, which had not been observed previously, consisted of small spots of colour of fairly uniform size distributed apparently at random over the aleurone layer (see Fig. 18.1(*a*)). The 12:3:1 ratio was evidently a modified 9:3:3:1 ratio,

and implied that the dotted character was due to a dominant gene inherited independently of that responsible for the otherwise colourless aleurone. Further breeding work confirmed this conclusion. The dominant gene responsible was called Dotted, *Dt*. It was found that a mutation had occurred at the A_1 locus to the recessive allele a_1 giving colourless aleurone, and that *Dt* interacted specifically with a_1 to give the dots, provided the genes A_2, *C* and

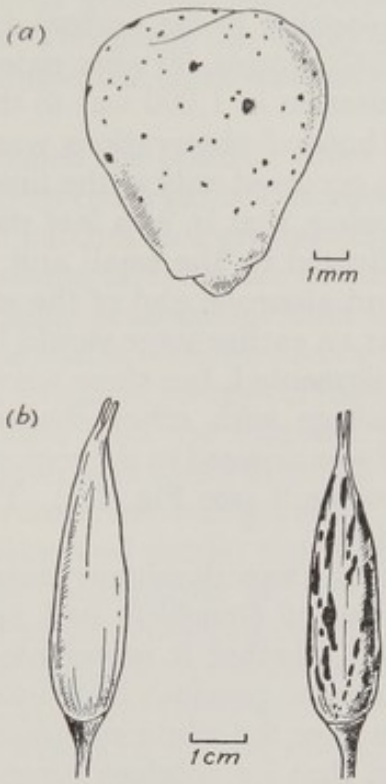


FIGURE 18.1 (*a*) A *Dotted* grain of *Zea mays*. The spots represent regions of purple anthocyanin pigment in the otherwise colourless aleurone layer of the endosperm.
(*b*) Flower-buds of *Oenothera blandina* showing the uniformly coloured calyx of a normal plant (left), and the mottled appearance (right) of an individual heterozygous for a pair of alleles affecting sepal pigmentation and with a structural change in this particular chromosome in the neighbourhood of that gene (from Catcheside, 1947).

R were also present. *Dt* was found to have no effect on recessive alleles at these other loci. It was found that *Dt* had been segregating previously in the strain of maize in which the mutation from A_1 to a_1 took place, but it had not been detected owing to the homozygosity for A_1.

The A_1 gene causes anthocyanin pigment to form in various other parts of the plant, provided the genotype is appropriate. These parts are the leaf-sheaths, the bracts surrounding the female inflorescence ('husks'), the stems ('culms'), the male inflorescence ('tassel'), the anthers and the pericarp. Rhoades (1938) found that *Dt* in the presence of a_1 and also of whatever other genes were necessary, caused small and narrow longitudinal stripes on the husks and culms; numerous small spots on the anthers or occasionally an entire anther coloured; and fine streaks or sectors on the pericarp. By

making crosses with pollen from the rare wholly purple anthers, he showed that the purple character was sometimes inherited and that it was quite stable and was due to the presence of the A_1 gene. It was evident that the dots or stripes in potentially coloured parts of *Dt* a_1 maize were arising as a result of mutation from a_1 to A_1. Rhoades found that *Dt* also stepped up the rate of mutation of a_1 to another allele called a_1^p which gives a paler anthocyanin than A_1. However, the rate of mutation from colourless to pale was much lower than from colourless to the normal dark pigmentation. Out of 80,955 aleurone dots on 2426 grains, 80 were pale and the remainder dark, implying that about 1 mutation in 1,000 was to the pale allele.

A striking feature of Rhoades' observations was that the mutations of a_1 induced by *Dt* apparently occurred only at the time when the A_1 gene might be expected to be functioning, that is, at a late stage of the development of each tissue. This was indicated by the small and relatively uniform size of the dots in the anthers and aleurone and of the stripes in the husks, culms and pericarp. Mutation at an earlier stage would be expected to give rise to whole regions or sectors pigmented, but these were rare or absent.

From studies of its linkage with other character-differences, Rhoades (1945) established that *Dt* was situated in the region of the knob at the end of the short arm of chromosome 9 (see Fig. 7.5). This knob is composed of heterochromatin (see § 18.3).

Muller (1930*a*) found that a new dominant mutation at the notched-wing locus in the *X*-chromosome of *Drosophila* had appeared following X-ray treatment. In order to test whether it represented a deficiency of a small chromosome segment, like some previous Notch mutants, he crossed it with white-eyed flies, since the gene for white eye-colour was known to be only about 1.5 map units from that for notched wings. Most unexpectedly, the notched-winged offspring had mottled eyes, with lighter and darker areas of diverse size and ranging in colour over the whole series of colours known for alleles of white eye. Muller found that the mottled character was inherited as an allele of white. He also found that the notching of the wings was irregular in its manifestation, both within and between individuals, and that the behaviour of the wing and eye characters was to some extent correlated, as though there was some peculiarity in a chromosome segment rather than in just one gene. Moreover, Muller found from study of linkage with other mutants that the notched-winged mottled-eyed flies had a structural change in the *X*-chromosome, such that the part of it containing the notched-wing and white-eye loci had been removed and attached to chromosome no. III. Four other independent occurrences of alleles at the white-eye locus giving mottled eyes were found following irradiation of sperm. Although these had normal wings, linkage studies revealed that in every instance there was a structural change involving the *X*-chromosome. These observations suggested that there might be a causal connection between the mottling and the structural change.

Such a connection was subsequently demonstrated with the white-eye alleles and a number of other genes in *Drosophila*. One of the clearest demonstrations was by Catcheside (1939, 1947) in *Oenothera blandina*. A plant homozygous for a gene giving light red sepals was self-pollinated using pollen

treated with X-rays. One of the progeny was found to have irregular patches of light red on otherwise green sepals. By examination of the chromosomes at meiosis, it was found that in this plant an interchange had occurred between segments of two of the chromosomes, and that one of these chromosomes was that which carried the gene for sepal colour. Through crossing-over in the interval between gene and point of interchange (recombination frequency about 1.7%), the sepal colour gene was subsequently transferred to a plant with normal chromosomes. This plant was found to show no variegation. It was inferred that the mottling of the calyx was due to a position effect and not to mutation.

Catcheside (1947) confirmed the position effect when he showed that an allele giving a uniformly deep red sepal colour also gave rise to a variegated calyx, this time of dark red patches on a green background, when transferred through crossing-over to plants carrying the interchange (see Fig. 18.1(*b*)). Moreover, a gene giving paler petal-colour than normal, which was linked to the sepal-colour gene (recombination frequency 8·5%), gave rise to variegated petals consisting of a mosaic of patches of the two shades of yellow when similarly transferred to plants with the interchange. Both sepal and petal colours became uniform again in progeny in which the genes concerned were restored to the normal chromosome configuration.

These experimental results, and similar data obtained by several authors from studies of variegated phenotypes in *Drosophila*, have usually been interpreted as evidence that the variegation was due to instability in the functioning of the gene and not to a mutational change of the gene itself. An alternative explanation is that mutations occur with high frequency in the tissues where the gene produces its specific effect, but that in other tissues, including the reproductive cell-lineage, the gene is stable.

There is good evidence for a connection between the occurrence of variegated phenotypes in *Drosophila* and variability in the extent of regions of the chromosome which stain differently from normal and consequently are called heterochromatic.

§ 18.3 Heterochromatin

One of the most striking features of chromosomes in many organisms is that certain parts of them stain differently from other parts. Heitz (1928), after studying the mitotic chromosomes of the liverwort *Pellia endiviifolia*, introduced the term *heterochromatin* to describe segments which stained more deeply than the majority (*euchromatin*). Subsequently, the use of the term heterochromatin was extended to include regions which stain differently from the rest, since it was found that the same region may stain more deeply at one period of the nuclear cycle and less deeply at another. Heterochromatic regions lack chromomeres, such as the euchromatic parts of a chromosome show in early prophase I of meiosis. Corresponding to this, the heterochromatic segments of the salivary gland chromosomes of *Drosophila* show an amorphous appearance without transverse bands. Small segments of heterochromatin occur at numerous points in the chromosomes, and are revealed by cold treatment prior to fixation. In addition, there are usually extensive

heterochromatic regions near the centromere of each chromosome. These centromere regions form an aggregate in the *Drosophila* salivary gland chromosomes (see Fig. 9.1), apparently as a result of a general attraction between the heterochromatin of the different chromosomes. Non-homologous pairing between heterochromatic segments has been observed at the pachytene stage of meiosis in *Zea*, and may be responsible for an association in inheritance between genes in different linkage groups observed by Longley (1945) in *Zea* (female side only), by Michie and Wallace (1953) in *Mus*, and by Shult and Lindegren (1958) in *Saccharomyces*.* Another feature of heterochromatin, discovered by Lima-de-Faria (1959), is that it appears to replicate after euchromatin (see Lima-de-Faria and Jaworska, 1968). Thus, Schmid (1963) has shown by tritium labelling that the late replicating regions of human chromosomes in tissue culture correspond to secondary constrictions.

In many organisms, chromosomes composed wholly of heterochromatin occur (review: Battaglia, 1964). They have been called supernumerary or *B*-chromosomes, and they usually vary in number in different individuals or populations of the species.

The heterochromatic regions appear to correspond to parts of the linkage maps largely without genes. Examples of this in *Drosophila* are the *Y*-chromosome and the centromere regions of the other chromosomes. The viability of individuals lacking a *Y*-chromosome, or lacking *B*-chromosomes, contrasts with the lethality of even small deficiencies from the normal or *A*-chromosomes. On the other hand, the heterochromatic regions are evidently not devoid of function even though they appear to lack normal genes. This is shown by the necessity for a *Y*-chromosome if male *Drosophila* is to be fertile, and by the existence of adaptations in the haploid generation of flowering plants favouring an increase in the number of *B*-chromosomes. The means by which such increase is brought about include failure of *B*-chromosomes to segregate normally at mitoses in the pollen or embryo sac, such that daughter nuclei in the germ-line receive more of them than do other nuclei. Selective fertilization by gamete nuclei with the larger numbers may also occur (for references, see Battaglia, 1964). Likewise, Rhoades (1942) found that in *Zea* heterozygous for a terminal heterochromatic knob in chromosome no. 10 the homologue with the knob reached the functional embryo-sac in 70% of meioses instead of the 50% expected if the orientation of the chromosome-pair on the meiotic spindle were at random.

From study of the number of sternopleural chaetae in stocks of *Drosophila melanogaster* differing only in their *Y*-chromosome, Mather (1944) found evidence for the occurrence in this chromosome of a number of genes having small and more or less additive effects on bristle number. It was inferred that heterochromatin is not inert but contains genes which are qualitatively different from those occurring in euchromatin.

Ris (1957) obtained electron micrographs of thin sections through heterochromatin in the nuclei of amphibians and found that its structure did not differ from that of euchromatin, except in the degree of packing of the fine threads seen. He also found that the chromomeres in leptotene chromosomes

* Camenzind and Nicklas (1968) found that, unlike Fig. 6.4, in spermatocytes of *Gryllotalpa hexadactyla* the *X*-chromosome regularly moves to the same pole as the larger component of an unequal bivalent; micromanipulation showed that the *X* is the active partner.

of *Lilium longiflorum* differ from the interchromomeric segments merely in the density of the folded and coiled threads. That heterochromatin should differ from euchromatin merely in the way its material is arranged is in keeping with some observations which suggest that under certain circumstances euchromatin can become heterochromatic.

Schultz (1936) studied the salivary gland chromosomes of a number of mutants of *Drosophila* showing mottled phenotypes, and found that in every instance the euchromatic region containing the genes in question had been transferred, as a result of a structural change, to the neighbourhood of heterochromatin. Moreover, Caspersson and Schultz (1938) found that this euchromatic region bordering on heterochromatin tended to acquire the appearance of heterochromatin, the transverse bands being lost. According to Schultz (1948) it appeared as if in some cells the heterochromatin modified the neighbouring euchromatin so that it resembled heterochromatin both in appearance and in lack of genetical activity, while in other cells there was no such modification, thus accounting for the mottled phenotype. The cytological observations thus appear to confirm the inference from the genetic data and suggest that the mottling results from the genes in question having been rendered inactive in some of the cells, the appropriate part of the chromosome having at the same time become heterochromatic in appearance.

§ 18.4 Controlling elements

McClintock (1950) discovered an interaction in *Zea* between mutable loci and the variegated phenotypes caused by position effect. A dominant gene which she had called Activator (*Ac*) appeared to cause mutation of certain other genes not linked to it. McClintock had established that *Ac* acted through an unlinked gene called Dissociation (*Ds*). In the presence of *Ac*, genes situated within about 5 map units on either side of *Ds* gave rise to a variegated phenotype similar to the effect of structural changes in *Drosophila* (described above) which bring euchromatin into proximity to heterochromatin. Moreover, in the presence of *Ac*, strains arose with different patterns of variegation, apparently as a result of mutation of *Ds* to new alleles.

A further remarkable feature of these genes such as *Dt*, *Ac*, and *Ds* which cause variability in the manifestation of other genes is that they occasionally become transferred to new positions either in the same or a different linkage group. This was shown for *Ac* and *Ds* by McClintock (1950), and for *Dt* by Nuffer (1955), who found *Dt* was situated in chromosomes nos. 6 and 7, respectively, in two South American strains of *Zea*, instead of in no. 9. With *Ac* and *Dt* such transposition does not affect the phenotype, but this is not true of *Ds*, since it is genes in proximity to *Ds* which give rise to variegation. The regions which *Ac*, *Ds*, and *Dt* occupy in the chromosomes appear to be heterochromatic.

Through the transfer of *Ds* to new positions, McClintock was able to confirm that on removal of *Ds*, the original phenotype was restored. As with the mottled phenotypes associated with transfer of euchromatin to the neighbourhood of heterochromatin described above, the effect of *Ds* on neighbouring genes is therefore usually interpreted as influencing gene action

rather than as causing mutation. However, McClintock (1950) has put forward the view that these position-effect variegations and those due to mutable loci are essentially one phenomenon, and she has given a striking series of parallels between them (McClintock, 1952). However, it is not clear how far the resemblances are due to the fact that both phenomena are associated with heterochromatin.

Another feature of *Ds* is that it is frequently associated with chromosome breakage or other structural changes at its locus. Alleles of *Ds* differ in the frequency of occurrence of such aberrations. Many of these structural changes could be the consequence of non-homologous crossing-over between different chromosomes or different parts of the same chromosome (cf. § 9.8).

In view of the evidence that *Ac*, *Ds*, *Dt*, and other factors like them (see reviews by Brink, 1964, and McClintock, 1965) are not normal genes, but appear to be abnormal states of a system affecting the manifestation of particular characters at a certain stage of development, McClintock (1957) has proposed the name *controlling element* for them. The characteristic features of such an element are that it does not occupy a fixed position in the chromosome complement, and that it appears to act only within the nucleus, serving as a gene mutator, or modifying or suppressing gene action.

§ 18.5 Controlling episomes

Dawson and Smith-Keary (1963) have studied instability in the suppression of leucine requirement in *Salmonella typhimurium* and found similarities to the mutable loci of chromosomal organisms. They have postulated that *controlling episomes*, having many of the properties of McClintock's controlling elements, are responsible for the genic instability which they have observed. As mentioned in § 16.3, the term *episome* was introduced by Jacob and Wollman (1958*b*) for genetic particles that can exist either free in the cytoplasm or integrated into the chromoneme. When free, such particles replicate independently of and usually more rapidly than the chromoneme. Examples of episomes include many bacterial viruses, and the sex factor in *Escherichia coli*. Dawson and Smith-Keary found from transduction experiments that certain mutants having a requirement for leucine could become leucine independent as a result of mutation at a neighbouring locus. Some of these mutants which suppressed the leucine requirement were found to give colonies on minimal medium showing great diversity in size. Evidence was found that this instability was due to genetic factors capable of undergoing transduction, and that in any particular unstable mutant, the instability was probably confined to a particular site within the suppressor locus. They suggested that a controlling episome became attached at this site within the gene and was retained there through successive backward and forward mutations which it caused. Loss of the controlling episome would restore stability. This hypothesis was supported by the discovery of a correlation of instability at the leucine-suppressor locus with stability of suppressors for proline requirement, and *vice versa*. It was suggested that the leucine and proline suppressors often competed for a single controlling episome.

A two-element system, such as *Ac* and *Ds*, or *Dt* and a_1, in *Zea*, has not been found in *Salmonella*, nor is there any evidence from *Salmonella* of a modification of gene expression, either of the gene to which the controlling episome becomes attached, or of neighbouring genes such as *Ds* affects in *Zea*. The mutability in the presence of *Dt* of genes such as a_1 when they occur in specific tissues, and the stability of the same genes when in other cells, would be accounted for on Dawson and Smith-Keary's hypothesis if in the development of the tissues where the mutation occurs, a controlling episome was transferred from *Dt* to a_1, while in other tissues there was no such transfer. If the episome contained a nucleotide sequence, the association between gene and episome might be through complementary base pairing. If there was a difference between them in nucleotide sequence, this would lead to mispairing, and if there was correction of heterozygosity in the DNA, a mutation might arise (cf. § 16.6). The high mutability would then be a consequence of a failure of the base sequence in the episome precisely to match that of the gene (or a part of it).

Both the hypothesis of mutation caused by controlling episomes, and of gene inactivation caused by proximity to heterochromatin (or to a controlling element within the heterochromatin) are discussed further in § 18.6, since they appear to have relevance to the control of differentiation.

§ 18.6 The control of differentiation

One of the fundamental problems in biology is how a diversity of types of cell can develop from a single cell, such as a fertilized egg. At one time it was thought that cell differentiation must be controlled from the cytoplasm, since mitosis ensures that daughter-nuclei are identical. However, this inference was based on the assumption that all the genes were active in every cell. This assumption is clearly no longer justified in view of the demonstration by Monod, Jacob, and collaborators that the synthesis of β-galactosidase in *Escherichia coli* is under the control of a regulator gene (see Chapter 15). Differentiation could therefore reflect the control of gene action.

Accepting that the specificity of the enzymes of an organism is inherited in the structural genes in its nuclei, enzyme activity might be controlled at any of 3 stages:

1. Control of enzyme synthesis at transcription, that is, messenger RNA formation from DNA.
2. Control of enzyme synthesis at translation, that is, polypeptide formation from messenger RNA.
3. Direct control of enzyme activity after synthesis.

Regulation at all these levels is known in bacteria. The mechanism of control, through induction and repression of enzyme formation, is not fully understood, but control at transcription is established as the action of the regulator gene, *i*, in the synthesis of β-galactosidase in *Escherichia coli*, while control at the level of translation appears likely in Harris and Sabath's experiments with penicillinase in *Bacillus cereus* (see § 15.7). Control of

enzyme activity, as distinct from synthesis, appears to be restricted to the first enzyme of a sequence, or if the biosynthetic pathway is branched, to the first enzyme of each branch, and to be brought about by the end-product of the reaction chain (cf. Umbarger 1962). Monod and Jacob (1962) proposed the name *allosteric inhibition* for this phenomenon, because the inhibitor is not a steric analogue of the substrate, and because, theoretically at least, there is no reason why substances other than the end-product of the reaction chain should not act as inhibitors. They pointed out that if the end-product of each of two independent biosynthetic pathways acted as an inhibitor of the enzyme activity for the first step in the other pathway, this would provide a basis for alternative stable states. However, they consider that a steady state would be insufficient to account for differentiation, which appears to require controlled changes in the capacity of individual cells to synthesize specific proteins.

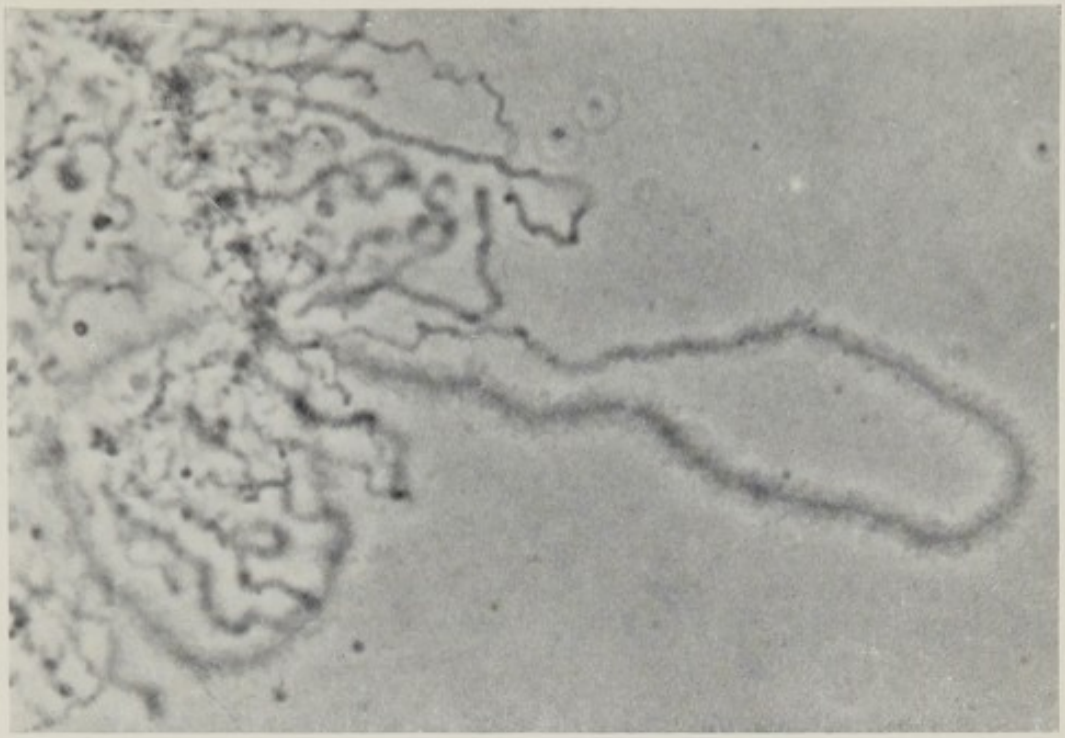
In chromosomal organisms, little is known in biochemical terms of the control of enzyme activity. If, as Monod and Jacob suppose, differentiation implies differential enzyme synthesis, there is the possibility, as in bacteria, of control at transcription or at translation. Control at transcription might be organized differently in chromosomal organisms compared with bacteria, owing to the occurrence of histones in association with the DNA.

A controlling mechanism implies a signal, and a transmitter and receiver. Control of gene action at the level of the transcription of DNA into messenger RNA requires the receiver of a signal to be in the nucleus. On the other hand, much of the protein synthesis of the cell occurs in the cytoplasm, so that control at the level of the translation of messenger RNA into polypeptide might occur in the cytoplasm. Equally, the transmitter might be either nuclear or cytoplasmic. Four possibilities are evident: intranuclear signals; extranuclear signals; and signals from nucleus to cytoplasm, and *vice versa*.

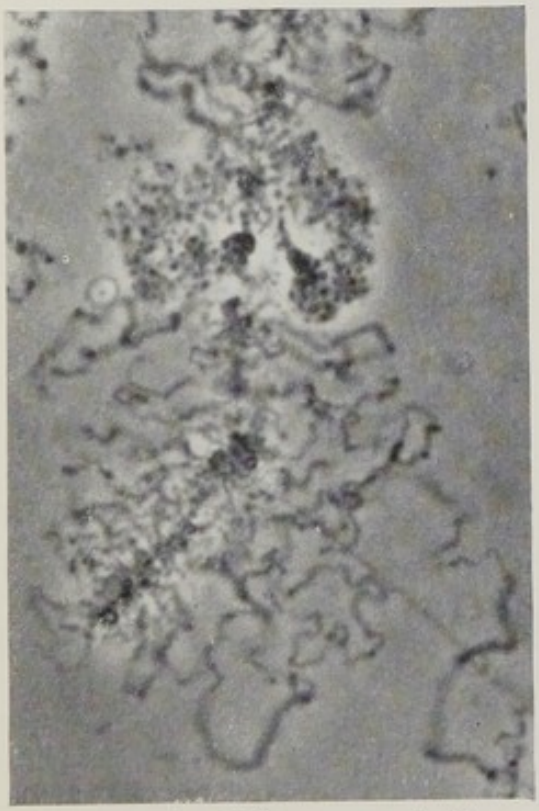
The occurrence of intranuclear signals in the control of gene action is exemplified by the regulator gene for β-galactosidase synthesis in *Escherichia coli* (see § 15.2), and by the evidence for controlling episomes in *Salmonella* and controlling elements in *Zea* (see §§ 18.4 and 5). In particular, Dawson and Smith-Keary's hypothesis of controlling episomes not only offers a possible explanation for mutable genes (see § 18.2), but it also suggests a mechanism for the control of differentiation. It appears significant that mutable genes often show mutation only at the time of development when the gene may be expected to be functioning. This suggests that the normal function of the episome might be to act as a signal for the formation of messenger RNA from that particular gene. If there is any truth in the suggestion that the mutability is due to the correction of heterozygosity in DNA (see § 18.5), in normal development the nucleotide sequences of gene and episome would presumably correspond, with the result that the gene would be stable.

Schultz (1948), using strains of *Drosophila* having chromosomal structural changes which brought genes into proximity to heterochromatin, found that the extent of the resulting variegated phenotypes associated with position effect (see § 18.3) was easily modified by genetic factors. In the presence of an extra *Y*-chromosome the development of a variegated phenotype did not

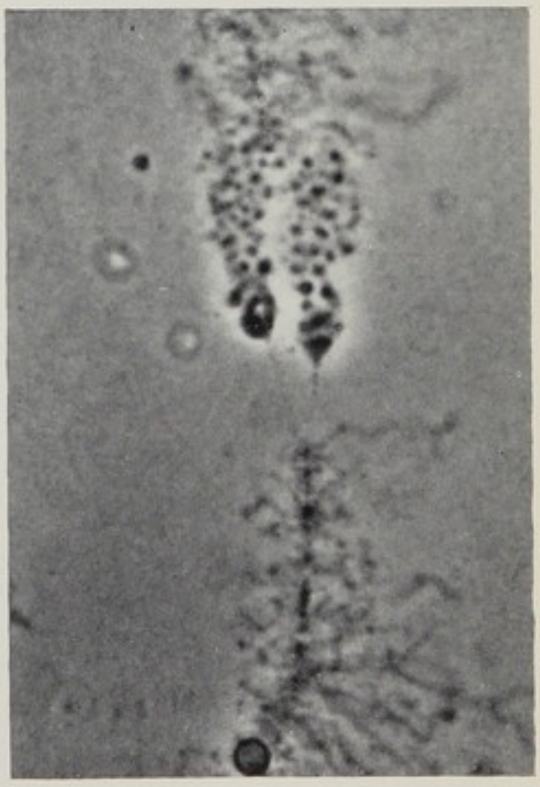
PLATE 7 Lateral loops of lampbrush chromosomes from oocytes of subspecies *cristatus* of the newt *Triturus cristatus*, showing the polarity in loop organization (see §18.7) (×700). *(Courtesy of H. G. Callan)*



(*a*) A large normal loop on chromosome I showing the greater thickness of matrix at one end of the loop compared with the other. The partner loop encircles the end of the chromosome.



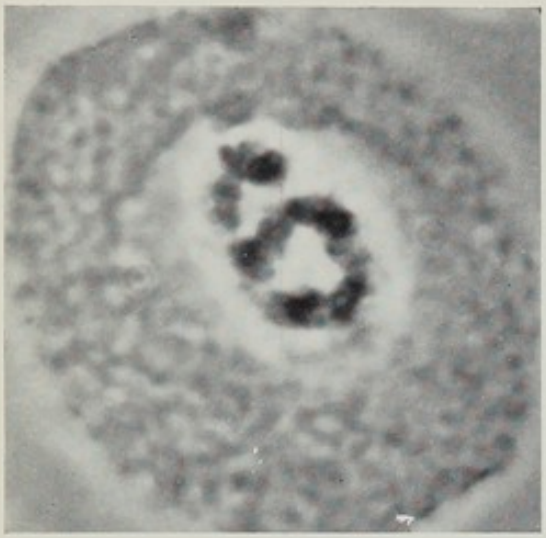
(*b*) The pair of giant granular loops which occur on the left arm of chromosome XII. These loops showed progressive labelling starting at the thin end when exposed to ^{3}H-uridine.



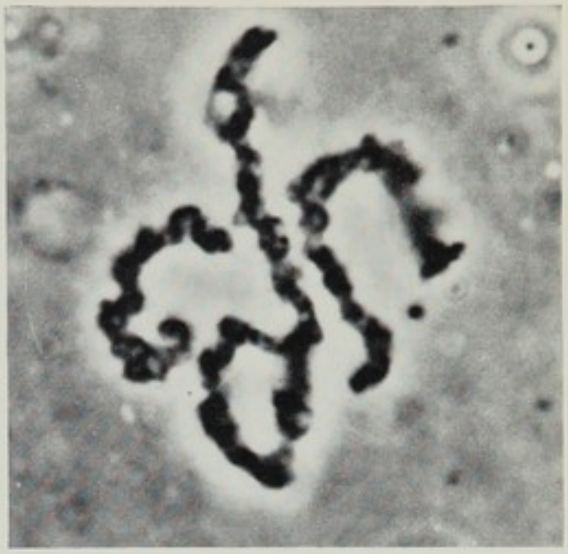
(*c*) The pair of loops seen in (*b*) forms a double bridge when the chromosome is stretched (see §17.6). These particular loops always have their thin ends directed away from the centromere.

PLATE 8 (*a*) and (*b*). Phase-contrast photographs of the loop which extends into the nucleolus from the nucleolar chromosome at the nucleolar organizer (see §18.7). In these examples the nuclei contain only one nucleolus and each copy of the nucleolar chromosome is associated with it.

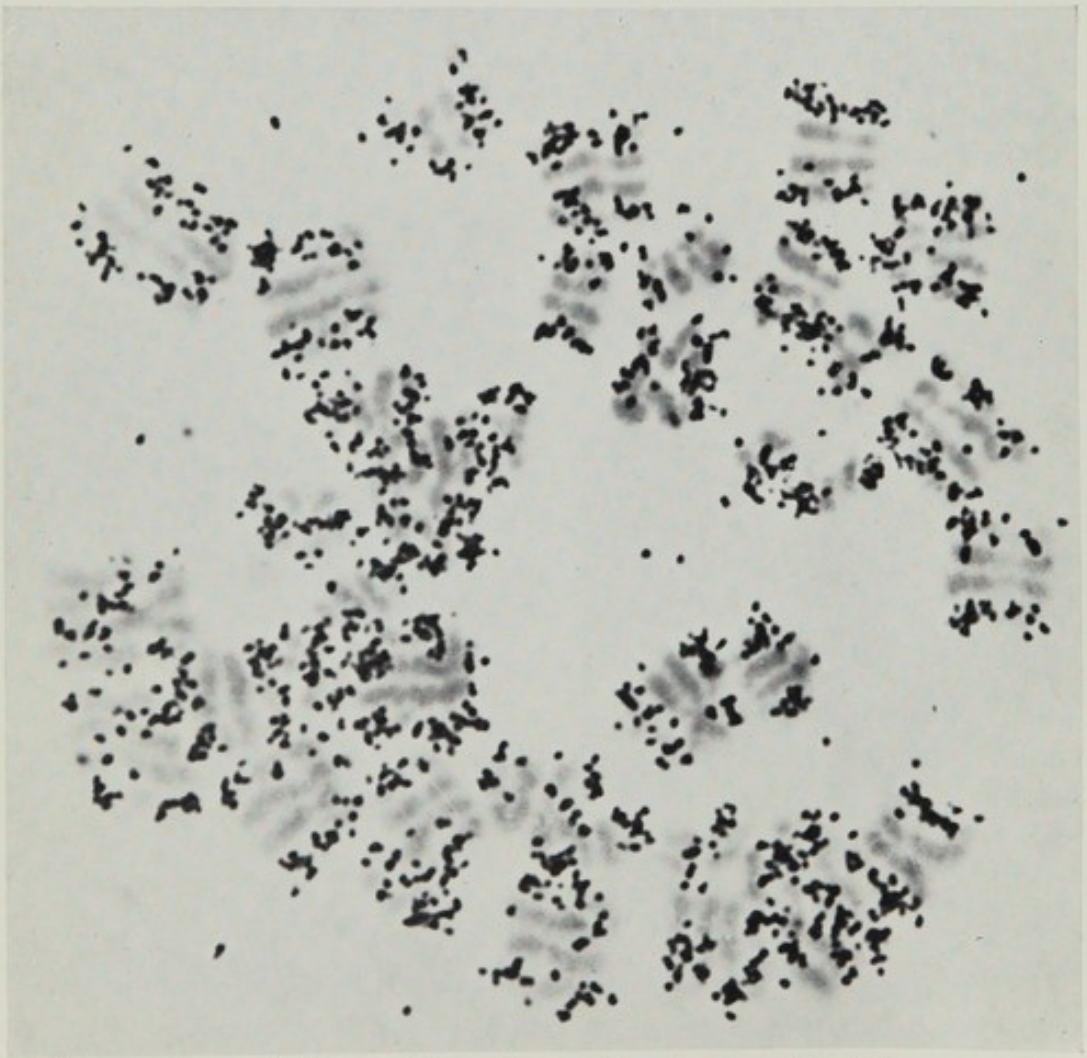
(*Courtesy of L. F. La Cour*)



(*a*) Two loops within the nucleolus of a diploid nucleus from a root-tip of *Ipheion uniflorum* ($\times$1650).



(*b*) Three filaments (broken loops) within the nucleolus of a triploid nucleus from endosperm of *Scilla sibirica* ($\times$1100).



(*c*) Autoradiograph of metaphase of an endoreduplicated cell from a culture of human peripheral blood cells, following treatment with ^{3}H-thymidine during the first of the two rounds of DNA synthesis ($\times$1650). The label is restricted to the two outer chromatids of each group of four that make up a diplochromosome, except where exchanges have occurred between chromatids (see §18.9).

occur and the chromosome region brought into proximity to heterochromatin by the structural change remained euchromatic. The influence of the relative numbers of the different chromosomes on development was referred to in § 6.8 with reference to the abnormality of trisomics. Some genes, for example, *Dt* in *Zea* (Rhoades 1936) and *w* in *Drosophila* (see § 9.1), show a dosage effect, that is, their action is dependent on the number of copies present in the nucleus, and so a trisomic individual will be expected to have a modified phenotype. However, the mechanism by which an extra *Y*-chromosome, which is more or less entirely heterochromatic, can modify the phenotype, is unknown.

A number of further observations indicate the receipt within the nucleus of signals affecting gene action, although their source (whether intra- or extra-nuclear) is usually not known. King and Briggs (1955) transplanted nuclei from *Rana pipiens* embryos at various stages of development into enucleated eggs. They found that the nuclei from certain tissues had lost certain potentialities of expression possessed by the original nucleus of the egg. It appeared that progressive and stable nuclear changes occurred during differentiation, even though, as later studies showed, there were no visible changes in the chromosomes.

§ 18.7 The master and slaves hypothesis

Gall and Callan (1962) studied the incorporation of tritiated uridine (the ribonucleoside of uracil containing ^3H) into RNA, and of tritiated phenylalanine into protein, in association with *Triturus* lampbrush chromosomes (the diplotene stage of meiosis in oocytes) and they found that RNA and protein synthesis occur on the lateral loops but not on the chromomeres. There was marked variation in intensity of labelling from one pair of loops to another, each of which has a characteristic morphology. These observations were in agreement with the hypothesis of Callan and Lloyd (1960) that each loop-pair is associated with the activity of one specific gene. Callan and Lloyd had reached this conclusion from their detailed studies of the lampbrush chromosomes in the oocytes of four subspecies of *Triturus cristatus*. They found that the presence or absence of particular loops of recognisable morphology showed Mendelian inheritance. Some individuals were heterozygous for particular differences (see § 8.13) and the relative frequencies in 22 individuals of the 9 combinations of homozygotes and heterozygotes for the presence or absence of a particular loop on chromosome X and another on chromosome XII (Pl. 6(*b*)) agreed with the Hardy-Weinberg equilibrium (§ 2.6). Support for the idea that a chromomere, or its counterpart the transverse band in salivary gland chromosomes, corresponds to a gene, has been obtained from studies of mutations in the white-notch region of the *X*-chromosome of *Drosophila melanogaster*, summarized by Beermann (1967).

In order to explain the discrepancy between the size of a lampbrush loop and the size of a gene as a nucleotide sequence coding for a polypeptide, Callan and Lloyd proposed that the lampbrush loop consisted of a series of

duplicate copies of one gene in tandem array. Moreover, they suggested that at each chromomere there was a 'master copy', mutations in which were somehow transferred to the other copies, from which messenger RNA was synthesized. Callan (1967) proposed a mechanism for this transfer of information from master to slaves. He suggested that the complementary chains of each slave in turn are separated from one another and paired with their respective complements in the master copy. If slave and master differ in nucleotide sequence as a result of mutation there will be mispairing in the master/slave hybrid DNA. Correction of mispairing is then assumed to occur, and moreover to take place in a defined direction, namely, from master chain to slave chain. In other words, it is postulated that the mispaired nucleotides of the slave chain are excised and replaced by nucleotides synthesized with the master chain as template. As explained in § 16.6, correction of mispairing arising from recombination is thought to occur widely, and moreover the direction of correction may be determined by features of the DNA other than the mispairing itself. Such external control of direction is an essential requirement for the matching of the slave nucleotide sequence to that of the master. Following the correction of the slave chains to correspond with the master, it is assumed that the slave chains separate from the master chains and anneal with one another. The whole process of matching slave to master would then be repeated with the next slave copy of the gene, and so on through the whole series of them.

Callan's hypothesis of matching slave nucleotide sequences to a master sequence makes the master and slaves hypothesis compatible with the results of studies on mutation and recombination, because a single master copy with means of transferring its nucleotide sequence to the slaves is equivalent in genetical terms to the single copy of the gene which the genetical data require.

Callan's matching hypothesis also explains an extraordinary polarity in the appearance of lampbrush loops, which he and Gall had observed. Callan and Lloyd (1960) reported that, irrespective of the characteristic texture of the protein matrix of particular loops, lateral loops had one end thin and the other much thicker at the points of insertion in the chromomere (Fig. 17.1(*d*) and Pl. 7(*a*)). They observed that when a chromomere was stretched until it broke (Fig. 17.1(*e*)), the polarity of any particular loop was always constant in direction with respect to the centromere of the chromosome (Pl. 7(*c*)), but either orientation was possible: 2 particular loops always had the thin end towards the centromere, and for 3 other loops the converse was true. To explain the loop asymmetry, Gall (1955) and Callan and Lloyd (1960) suggested that the loop axis was spun out from the chromomere at the thin end and returned to it at the thick end, for example, clockwise movement in the lower loop of Fig. 17.1(*f*). Such movement would explain the greater accumulation of gene product on the one side of the loop than the other. Support for this idea of polarized loop extension and retraction was obtained by Gall and Callan (1962) who found from their RNA labelling with ^{3}H-uridine that some loops (cf. Pl. 7(*b*)) showed progressive labelling starting at the thin end but not reaching the thick end for 10 days. The

matching hypothesis explains this movement of the loops by supposing that the master copy is at one end of the tandem array and that the thin end of the loop is the point where slaves emerge from the chromomere after they have been matched against the master copy.

Keyl's discovery of the successive doubling in evolution of the DNA content of individual chromomeres, probably as a result of doubling the length of the DNA molecule or molecules which they contain (§ 17.8), implies on the master and slaves hypothesis a doubling of the number of slave copies of particular genes. The number of copies of a gene in a large chromomere might be very high. Allowing for the feeding back of the lampbrush loop into the chromomere, Callan (1963) estimated that one of the largest loops in *Triturus cristatus*, together with its chromomere, might contain 1–2 mm. of DNA. A gene of 1000 to 1500 nucleotide-pairs would occupy 0·34 to 0·5 μ of DNA, so the largest *Triturus* chromomere might contain 2^{12} or more copies of the gene.

Edström and Beermann (1962) have shown that the RNA associated with diffuse areas or puffs at specific points in the giant chromosomes of the salivary glands of *Chironomus tentans* has a base composition which is characteristic for each puff. Beermann (1961) has shown that in *C. pallidivittatus* granules are secreted from particular cells in the salivary gland, and that in these cells but not in others, puffing occurs at the position in the appropriate chromosome of a gene concerned with production of the granules (see Fig. 9.1(*b*) and (*c*)). In hybrids with *C. tentans*, which lacks the granules, the *C. pallidivittatus* chromosome alone shows the puff. The inference from these experiments is that loop-formation in lampbrush chromosomes, and puffing in salivary gland chromosomes are associated with the activity of specific genes. Since there is evidence for characteristic patterns of puffing which vary with the stage of development and with the tissue, Beermann's findings constitute good evidence that differentiation is associated with differential gene activity. Moreover, Clever (1961) has shown with *Chironomus* larvae that injection of ecdysone, which causes premature pupation, initiates within a few minutes puffing at a specific locus in one of the salivary gland chromosomes, to be followed by a whole series of puffs corresponding in position and time sequence to those occurring at normal pupation. There is an obvious resemblance between lampbrush loops and the puffs of salivary gland chromosomes, and it seems that both represent the activity of specific chromomeres. According to Callan's master and slaves hypothesis the puff would represent a set of copies of one gene in each of the many parallel strands making up the giant chromosome.

Beerman (1965, 1967) put forward an alternative explanation for puffs and lampbrush loops. He suggested that the newly-made RNA must be packaged in protein before it can leave the chromosome, and that a function of the DNA axis of the loop was to provide a substrate for binding and stabilizing the messenger, in readiness for its association with transport protein. On Beermann's hypothesis the master segment at the base of a loop is active in transcription and the remainder of the loop is concerned primarily, at least, with packaging, whereas on Callan's hypothesis the

master copy does not take part in transcription, which is confined to the slaves in the loop.

Attardi, Parnas, Hwang and Attardi (1966) and a number of other authors have discovered giant-sized RNA molecules in the cells of vertebrates, having sedimentation coefficients of 30*S* to 80*S* or more. This RNA has a composition similar to that of the total DNA of the organism, and may possibly be derived from a set of copies of one gene. The significance of the duplication of genes within chromosomes is presumably to allow a higher rate of messenger synthesis.

Fincham (1967) suggested that the master and slaves hypothesis may account for some of the features of mutable genes (§§ 18.2 and 4). Certain mutations or structural changes in the master copy might interfere with the correction of mispairing in the process of matching the slaves to the master, and thus also affect transcription. The gene could then be inactive, although the slaves were non-mutant. The mutability would then lie in the possibility, which might arise in various ways, of one of the slaves taking over the function of the master-copy.

It is probable that the nucleolus represents visible evidence of the activity of a specific gene or group of genes, since the base composition of its RNA agrees with that of the ribosomes and has led to the suggestion that the nucleolus is the site of ribosome formation. Elsdale, Fischberg and Smith (1958) discovered a female *Xenopus laevis* (Clawed Toad) in which half the offspring possessed only one nucleolus in each nucleus instead of the normal two. These heterozygotes for the nucleolar deficiency were of normal phenotype and on intercrossing gave a 1:2:1 ratio of individuals with 2, 1, and 0 nucleoli, respectively, per nucleus. The homozygotes lacking nucleoli died as young larvae. Brown and Gurdon (1964) showed that the embryos lacking nucleoli were unable to synthesize either of the major RNA molecules which occur in ribosomes. These have sedimentation rates of 18*S* and 28*S*. The survival of the homozygotes to the tadpole stage appeared to depend on the use made of ribosomes inherited cytoplasmically from the mother. Wallace and Birnstiel (1966) showed from DNA–RNA hybridization studies that the genetic defect in the nucleolus-deficient *Xenopus* is a deletion of at least 95% and possibly the whole of the DNA which is complementary to ribosomal RNA. They found that in normal *Xenopus* 0·07% of its DNA hybridizes with 28*S* ribosomal RNA and 0·04% with 18*S*. With 6 pg of DNA per erythrocyte nucleus, they estimated there were about 800 copies per haploid set of chromosomes of the genes for the ribosomal RNA components. Brown and Weber (1968), using an improved technique involving density-gradient centrifugation in caesium chloride to fractionate the DNA, followed by hybridization of RNA to DNA immobilized on nitrocellulose filters, concluded that each nucleolar organizer contains 450 copies of an operon consisting of the 28*S* and 18*S* genes. Other nucleotide sequences occurring between these genes and amounting to about 40% of the ribosomal RNA precursor, appear to be discarded during ribosome formation.* There are estimated to be at least 27,000 copies of the gene for the 5*S* RNA

* Similar results were obtained by Birnstiel *et al.* (1968).

component of the ribosome, and these 5*S* genes have not been lost in the nucleolus-deficient *Xenopus*.

Ritossa and Spiegelman (1965) hybridized *Drosophila* ribosomal RNA with the DNA of strains of *Drosophila* having from 1 to 4 doses of the nucleolar-organizer region, which is in the *X*- and *Y*-chromosomes. The abnormal doses were from stocks which had either a duplication or a deletion of the nucleolar-organizer region of the *X*-chromosome. The hybridization was achieved by incubating the denatured (single chain) DNA with the RNA for 7–10 hours at 65°C. It was found that the amount of DNA which would hybridize with ribosomal RNA was proportional to the number of nucleolar organizers present. It was inferred that all the DNA which specifies nucleotide sequence in ribosomal RNA is situated in the region of the nucleolar organizer. This supports the notion that the nucleolus is the site of ribosomal RNA synthesis. It was found that no less than 0.27% of the DNA of a normal fly could be hybridized with ribosomal RNA. Ritossa and Spiegelman favoured the idea that the genes for ribosomal RNA are repeated consecutively along the chromosome 100 or more times.* Ritossa, Atwood and Spiegelman (1966*b*) made the surprising discovery that the gene in *Drosophila melanogaster* for bobbed bristles (*bb*), which is situated in heterochromatin near the centromere (see Fig. 8.10), is none other than the nucleolar organizer. Different *bb* mutants showed from 30% to 55% of the normal amount of DNA complementary to ribosomal RNA.

Matsuda and Siegel (1967), by the hybridization technique, estimated there were 750 copies of the genes for ribosomal RNA per haploid chromosome set of *Nicotiana tabacum*.

The duplication of the genes for ribosomal RNA in *Drosophila*, *Xenopus* and *Nicotiana* is in agreement with the experimental results obtained by McClintock (1934) with *Zea mays* and Beermann (1960) with hybrids of *Chironomus tentans* and *C. pallidivittatus*. These authors discovered that when the nucleolar organizer is broken into two parts by X-irradiation, each part can fulfil the functions of a complete organizer and sustain normal development.

The occurrence in chromosomal organisms of duplicate copies of the genes for ribosomal RNA at the nucleolar organizer gives support to the master and slaves hypothesis of chromomere organization, because Callan (1966) has found that the nucleolar organizer locus in the lampbrush chromosomes of *Ambystoma mexicanum* is organized in the same way as other chromomeres. Moreover, La Cour and Wells (1967) found that in the nucleoli of *Ipheion uniflorum* a loop about 20 μ long, and with a DNA axis, extends from the nucleolar organizer (see Plate 8(*a*) and (*b*)).

On the other hand, duplication of genes for ribosomal RNA is also known in bacteria: Yankofsky and Spiegelman (1962) found that 0·2% of the DNA of *Escherichia coli* hybridized with 23*S* ribosomal RNA, implying about 10 copies of the gene, and Smith *et al.* (1968) estimated that in *Bacillus subtilis*

* Quagliarotti and Ritossa (1968) obtained evidence that there are equal numbers of the 18*S* and 28*S* genes and that, as in *Xenopus*, each 18*S* gene alternates with a 28*S*.

there were also about 10 copies of the genes for the 16*S* and 23*S* ribosomal RNAs, and about 4 copies of the gene for the 5*S* component.

There seem also to be duplicate copies of the genes for transfer RNAs. Ritossa, Atwood and Spiegelman (1966*a*) estimated that in *Drosophila melanogaster* 0·015 % of the haploid DNA content of 0·2 pg hybridized with transfer RNA, or enough for about 750 *t*RNA genes. The minimum number of different transfer RNAs is 32, but a larger number is probable (cf. § 14.15). It is evident, however, that there is considerable duplication of the 40 or 50 *t*RNA genes likely to be present. Goodman *et al.* (1968) found two identical tyrosine *t*RNA genes in *E. coli* (see § 14.17) and Gilmore *et al.* (1968) found 8 genes for tyrosine *t*RNA in *Saccharomyces cerevisiae*; these genes were not closely linked, however.

The identification of genes for ribosomal RNA and transfer RNAs implies that some genes are concerned in specifying nucleotide sequence in RNA for its own sake, rather than as coded information for polypeptide synthesis. Since polypeptide-determining genes and operons are defined by one polypeptide and one messenger, respectively, RNA-determining genes are presumably best defined in terms of the ultimate RNA molecules transcribed from them, even if larger segments were formed initially.

§ 18.8 The cycloid model for the chromosome

Callan (1967) drew attention to a feature of chromosome organization which seems to be of fundamental importance, namely, that lampbrush loops do not take part in crossing-over. There are three lines of evidence which point to this conclusion. First, he had never observed chiasmata in the loops. Secondly, the chromomeres are conspicuous in early prophase of meiosis when crossing-over takes place; they are too densely folded for the occurrence of homologous pairing at the molecular level, such as crossing-over requires. It is significant that the greater part of the material of homologous chromosomes lies outside the synapton at pachytene (see § 8.20 and Plate 4(*a*)). Thirdly, accepting the master and slaves hypothesis, if pairing took place at meiosis between slaves, it would be expected often to occur with the homologues relatively displaced, for example, slave no. 5 from one

FIGURE 18.2 Diagrams to show a structure for the chromosome in keeping with recombination data and with the work of Callan and Gall on lampbrush chromosomes and of Keyl on dipteran salivary gland chromosomes. In (*a*) is shown the normal state of the chromosome with sets of duplicate genes inserted in the DNA axis to give the form of an irregular cycloid, while (*b*) represents the situation at pachytene of meiosis with the duplicates removed as rings of DNA. Eight master-genes are shown, each with 2, 4, 8 or 16 slave-copies. Much larger numbers of copies would be present in organisms with high DNA contents to their chromosomes. Transverse lines and arrows indicate the ends of each gene copy and its orientation, with double lines separating unlike genes. The letter O indicates the hypothetical position of the recombination-operator of each gene (see § 16.7). The loops or rings of DNA would correspond to chromomeres.

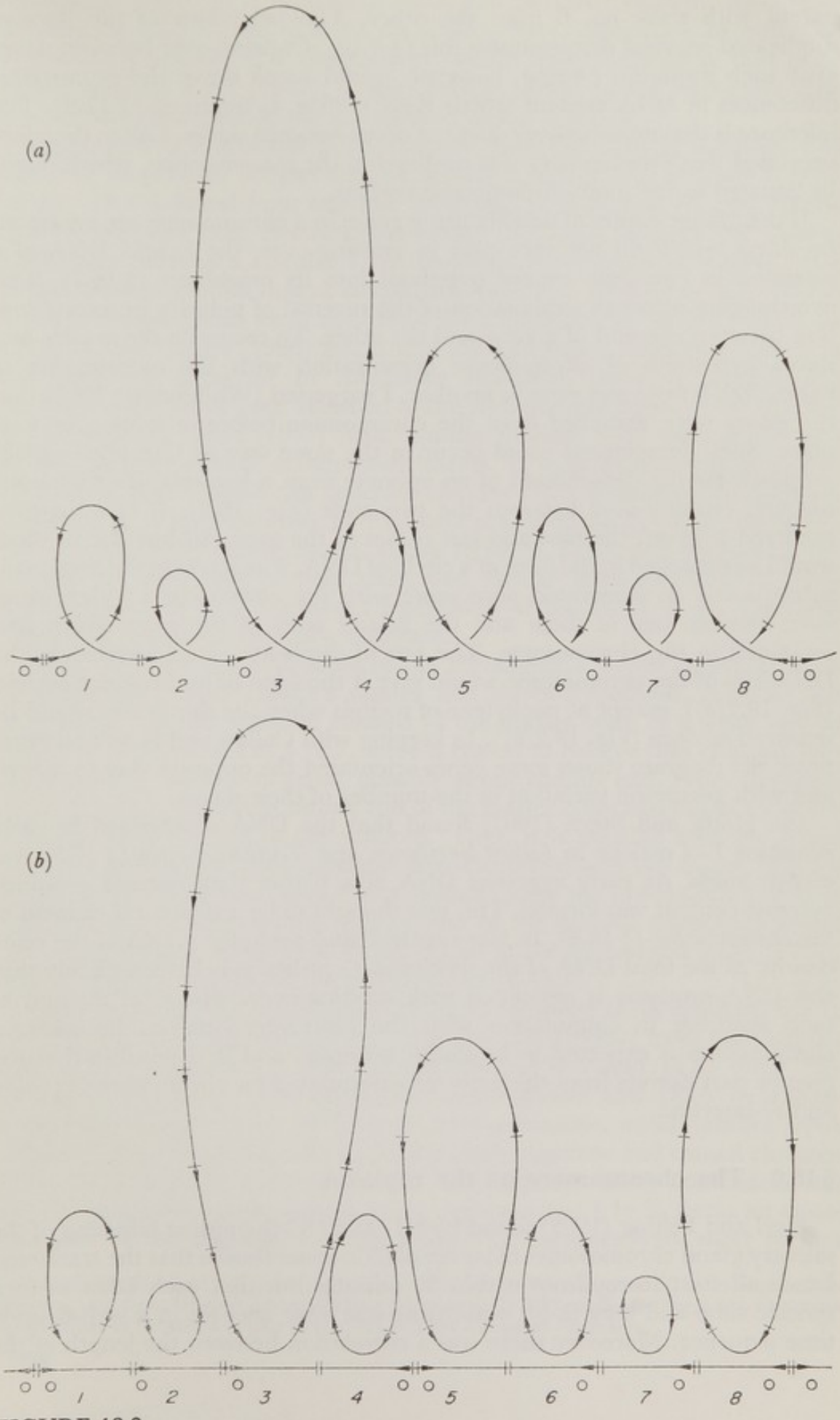


FIGURE 18.2

parent with slave no. 6 from the other. The behaviour of the Bar-eye duplicated segment demonstrates this (§ 9.10). Crossing-over between slaves after such displaced pairing, however, would break down the geometrical differences in DNA content which Keyl (1965*a*, *b*) observed (§ 17.8). The inference is that crossing-over does not occur between slaves. Callan therefore suggested that crossing-over was confined to the master-copies, which might be situated in the inter-chromomeric regions.

If the master-copies of neighbouring genes in a chromosome are separated by slaves which do not take part in crossing-over, the hybrid DNA of a crossover in one gene cannot overshoot into its neighbour (§ 16.7). This overshooting offers an explanation of the reversal of polarity in recombination between one end of a gene and the other. To reconcile the master and slaves hypothesis of chromomere organization with the overrunning of hybrid DNA from one gene to another, I suggested (Whitehouse, 1967) that the slaves were detached from the chromosome before crossing-over took place. Such detachment could occur in the same way as Campbell (1962) proposed for the detachment of an episome from a bacterial chromoneme, namely, crossing-over between the two ends (Fig. 16.4). If crossing-over occurred between the first and last copies of the gene, all but one of them would be detached in the form of a circle of DNA. The slaves in the ring could subsequently be integrated once more with the chromosome by crossing-over between one of them and the master copy of the gene which had remained in the chromosome. The sets of duplicate genes inserted in the DNA axis of the chromosome would give it the form of an irregular cycloid (Fig. 18.2(*a*)), except at pachytene of meiosis when the duplicates would be removed as rings (Fig. 18.2(*b*)). In keeping with Callan and Keyl's observations, the diagram shows some genes orientated the opposite way to others, and with geometric variation in the number of their slaves.

Ito, Hotta and Stern (1967) found that the DNA synthesized in early prophase I of meiosis in *Lilium longiflorum* and *Trillium erectum* (§ 16.5) was of two kinds. At early zygotene DNA of a higher than normal guanine–cytosine content was formed. This was thought to be a structural element of the chromosome (§ 18.9). In late zygotene and pachytene DNA of the same density as the total DNA of the species was synthesized. It seems likely that this DNA synthesis is associated with crossing-over. The total amount of such synthesis in conjunction with the crossovers between homologous chromosomes is expected to be slight, however, and it is possible that the greater part derives from the crossovers postulated for chromomere excision and re-insertion.

§ 18.9 The chromomere as the replicon

Keyl and Pelling (1963) found by ^{3}H- and ^{14}C-thymidine labelling of the salivary gland chromosomes of larvae of *Chironomus thummi* that the transverse bands all start to replicate within 30 minutes but that each takes its own specific time and they finish replicating one after another in a well-defined time sequence. Moreover there was a correlation between the length of the

replicating period of a transverse band and its DNA content. These discoveries, in conjunction with Keyl's finding that duplication in evolution of the DNA content of a chromomere occurs independently of that of its neighbours, led Keyl (1965*a*, *b*) and Pelling (1966) to the important conclusion that the chromomere corresponds to the replicon.

Support for this idea has been obtained from the study of nucleoli. The nuclei of amphibian oocytes contain several hundred free nucleoli (Pl. 6). Miller (1964*a*) found from enzymic treatment with pepsin, trypsin, RNase and DNase, and from electron microscopy that each nucleolus of *Triturus pyrrhogaster* contains a circular DNA molecule surrounded by matrix. This construction resembles that of lampbrush loops and led him to suggest that a single chromosomal locus may replicate independently of the rest of the chromosome. Callan (1966) found that the lampbrush loop at the locus of the nucleolar organizer in *Ambystoma mexicanum* resembles ring-shaped nucleoli which occur both attached to the chromosome at this point and free in the nucleus. Kunz (1967) made similar findings with the free nucleoli in the oocytes of some orthopteran insects. It appears from these studies that the nucleolar locus can generate circular DNA molecules, presumably consisting of a tandem array of duplicate copies of the genes for ribosomal RNA.* McLeish (1964) found about 10 pg of DNA per nucleus in the nucleoli of *Vicia faba* root-tip cells, and similar large amounts in other flowering-plants, so independent replication of the DNA of the nucleolar organizer may be of general occurrence.

Keyl and Pelling's hypothesis of the chromomere as the replicon, taken in conjunction with Callan's master and slaves hypothesis, leads to the conclusion that the replicon consists of the master copy of a gene and its slaves. As pointed out in § 16.7, there is reason to believe that crossing-over is initiated at the ends of genes, possibly at one specific end. From the argument of § 18.8 this would be one end of the master-copy and might therefore coincide with the end of the replicon. Thus, it is possible that the initial step in crossing-over is not breakage of nucleotide-chains but failure of DNA ligase to join chains at the end of a replicon after the pre-meiotic round of DNA synthesis. This would explain one of the earliest discoveries about crossing-over, namely, that it always occurs at the four-strand stage (§ 8.6), and this is true also of mitotic crossing-over (§ 13.3).

Direct support for this idea of a failure to complete DNA synthesis before meiosis has been obtained by Ito, Hotta and Stern (1967) from their studies on pollen-mother-cells of *Lilium longiflorum* (§§ 16.5 and 18.8) using ^{32}P-phosphate labelling, density-gradient centrifugation and DNA hybridization. They found that the addition of deoxyadenosine, which inhibits DNA synthesis, stopped meiosis when added in early zygotene and caused chromosome fragmentation when added in mid-zygotene. They concluded that the *GC*-rich DNA synthesized in early zygotene (see § 18.8) forms an essential part of the chromosome and consists of comparatively short regions

* Gall (1968) found differential synthesis of the genes for ribosomal RNA at oogenesis in *Xenopus*, and from ^{3}H-thymidine labelling Macgregor (1968) concluded there was successive duplication of detached copies of the DNA of the nucleolar organizer.

interspersed along the entire length of the chromosomes. These regions would be synthesized in mitotic interphase, but before meiosis their synthesis would be delayed until zygotene. In support of this hypothesis, Stern and Hotta (1967) found that pollen-mother-cells transferred to culture medium when in premeiotic G2 were diverted to mitosis instead of meiosis, and a small amount of *GC*-rich DNA synthesis then occurred before the mitosis; such synthesis was not found before a normal mitosis. It is evident that the delayed synthesis of segments of the DNA is peculiar to meiosis.

Assuming that the chromosome is single-stranded and with a continuous DNA axis, it is likely that the replicons making up the chromosome have staggered ends, that is, the breaks in the chains needed for replication do not occur opposite one another but some nucleotides apart. This would prevent breakage of the whole molecule and would be comparable to the 'sticky ends' in phage λ of *Escherichia coli*, where the 5′-terminal chain has been found to extend about 15 nucleotides beyond the 3′-terminal chain at both ends. The protruding chains at each end of λ are believed to have complementary nucleotide sequences, thus allowing the ends to join to give first a hydrogen-bonded and then a covalently-closed circle of DNA (Wu and Kaiser, 1967; Gellert, 1967; and see Figs. 14.6 and 16.4). In the chromosomes of higher organisms, the protruding chain at the right-hand end of each replicon would need to be complementary to the protruding chain at the left-hand end of the next replicon to the right. The *GC*-rich DNA synthesized in early zygotene might represent delayed formation of the protruding ends of the chains which had been synthesized at the previous S period.

Daughter-chromatids sometimes fail to separate at mitosis and this process, called *endoreduplication*, can be induced by desacetyl-methyl-colchicine. It gives rise to associations of 4 chromatids called *diplochromosomes*. Walen (1965) with cultures of kidney cells of the marsupial *Potorous tridactylus*, and Schwarzacher and Schnedl (1966) with human blood cells in culture, independently discovered that diplochromosomes regularly have the outer chromatids labelled and the inner not, after one replication in the presence of ^{3}H-thymidine followed by another without the label (Pl. 8(*c*)). The distinction between inner and outer chromatids is possible because the four lie in one plane. The labelling pattern would be accounted for if at each replication the 2 new sub-units were formed on the outside of the 2 old ones, that is, the opposite of the situation shown in Fig. 12.2(*b*). According to the single-strand theory of the chromosome, the sub-units are individual nucleotide-chains of DNA. A new chain can be on the outside of an old one only at specific points, however, because old and new form one double helix. These specific points are evidently not the centromeres because Herreros and Giannelli (1967) found the characteristic outer-chromatid labelling pattern in acentric diplochromosomes. It seems likely that the points where the new pair of chains regularly lie on the outside of the old pair are at the junctions of the replicons because, as indicated in § 12.7, it already seemed likely that a mechanism existed for joining new chain to new, and old to old, at these points.

The discovery by Huberman and Riggs (1968) of pairs of diverging replication-forks in *Cricetulus* chromosomes (§ 12.7) suggests that replication of the stretches of DNA corresponding to two neighbouring chromomeres may be initiated simultaneously at a common starting-point between them. Two kinds of replicon junctions would then be predicted, corresponding to the origin and the terminus, respectively, of the replication of the adjacent chromomeres. It may be significant that Haut and Taylor (1967), from study of DNA replication in *Vicia faba* root-tips, using 5-bromouracil as a density label, obtained evidence for short primer segments of two kinds, with different thymine contents.

Knowledge of the structure of replicon junctions, which are presumably situated in the interchromomeric regions, seems to be essential for an understanding of both chromosome replication and crossing-over.

§ 18.10 Chromosome inactivation

Lyon (1961, 1962) has postulated that in female mammals one of the two *X*-chromosomes is inactive. She has suggested that in the human embryo inactivation occurs at about the 12th day after fertilization, and that the choice is made independently in each cell, but once made is maintained indefinitely not only in these nuclei but also in all nuclei derived from them during later development. Cytological studies have shown that one *X*-chromosome is more tightly coiled than the other and replicates later. As a consequence of the random imposition of inactivity, female mammals will be mosaics for any sex-linked characters with local gene action for which they are heterozygous. This is exemplified by a number of mottled phenotypes known in mice (*Mus musculus*), and by the tortoiseshell cat (*Felis catus*).

A feature of Lyon's hypothesis of great interest is that the inactive *X*-chromosome shows all the pecularities of heterochromatin, that is, differential staining properties, late replication, and genetical inertness. Evidence was given in § 18.3 that the variegated phenotypes which occur when genes are transferred, as a result of a structural change, to the neighbourhood of heterochromatin, are associated with a change in the appearance of the euchromatic segments so as to resemble heterochromatin. These observations naturally raise the question of whether heterochromatin is always to be regarded merely as inactive (or largely inactive) euchromatin. Whatever the nature of heterochromatin, it is evident that, if Lyon's hypothesis is correct, a controlling mechanism must exist whereby the whole *X*-chromosome is integrated and all its genes are rendered inactive together.

Cattanach and Isaacson (1967) studied the behaviour of autosomal genes in *Mus* when these were transferred to the *X*-chromosome as a result of a translocation. From the occurrence of mottled phenotypes for genes in the transferred segment of the autosome, they inferred that inactivation of the *X*-chromosome showed a spreading effect analogous to that known for structural changes in *Drosophila* when euchromatin is placed next to heterochromatin (§ 18.3). The greater the distance of an autosomal gene from the point where the autosome was joined to the *X*-chromosome the less often

that gene was found to be inactivated. The extent to which the inactivation spread from the *X*-chromosome to the autosome appeared to be determined by a controlling element situated in the *X*-chromosome. This gene existed as a number of different alleles giving inactivation to different distances. Mutations of this gene were frequent. McClintock found similar mutations with the controlling elements which she studied in *Zea* (see § 18.4). Two distinct processes may be at work in the *X*-autosome translocation: first, inactivation of an *X*-chromosome, and secondly, extension of this inactivation into the autosomal segment. It seems more probable, however, that the controlling element is responsible for both processes.

Grüneberg (1967) questioned the validity of Lyon's hypothesis. From study of the phenotypes of female *Mus* heterozygous for various sex-linked genes, or for autosomal genes translocated to the *X*, he concluded that gene action was partially inhibited in both *X*-chromosomes, the genes in each acting at half-strength. He considered that the experimental results of others also conflicted with the single-active-*X* hypothesis, but could be explained by two partially active *X*s. There seems to be agreement (*a*) that a controlling element situated at a certain point in the *X*-chromosome can cause an inhibition of gene action to spread along the chromosome from this point; (*b*) that if a segment of an autosome is attached to an *X*-chromosome, the inhibition can extend into it, though with diminishing intensity; and (*c*) that the controlling element is activated only if two *X*-chromosomes are present. There is difference of opinion, however, whether the controlling element in both *X*-chromosomes is then activated (Grüneberg), or in one of them only (Lyon), and whether the inhibition of gene action is partial (Grüneberg) or total (Lyon). Grüneberg's hypothesis conflicts with the cytological evidence that one *X*-chromosome appears heterochromatic and the other euchromatic. Lima-de-Faria and Jaworska (1968) have emphasized, however, that the distribution of heterochromatin can differ from one tissue to another: in spermatocytes of the grasshopper *Melanoplus differentialis* the *X*-chromosome is heterochromatic and replicates late, whereas in spermatogonia it is euchromatic and replicates at the same time as the autosomes.

Henderson (1964), using tritiated uridine, studied RNA synthesis in association with the meiotic chromosomes of males of the locusts *Schistocerca gregaria* and *Cyrtacanthacris tartarica* and the grasshopper *Chorthippus brunneus*. He found that with the autosomes RNA synthesis occurs at all stages except when the chromosomes are strongly contracted (metaphase and anaphase, I and II). The single *X*-chromosome was found to remain tightly coiled and heavily staining throughout meiosis (see Plates 2 and 3), and to show no RNA synthesis, indicating that the activity of its genes is under generalized control (see Pl. 2(*f*)).

That histones may play a part in the control of gene action is suggested by the discovery of Izawa, Allfrey, and Mirsky (1963) that the addition of arginine-rich histones to *Triturus* lampbrush chromosomes stops the formation of RNA in association with the lateral loops, and leads to their retraction into the chromomeres. Bonner, Huang, and Gilden (1964) found that nucleoprotein extracted from *Pisum sativum* will bring about the synthesis of proteins

in vitro. They used the protein synthesizing system derived from *Escherichia coli* and developed by Nirenberg and Matthaei (see § 14.12). Bonner *et al.* discovered that the proteins synthesized with this system, using nucleoprotein from the developing cotyledons as primer, include a globulin characteristic of the cotyledons. The nucleoprotein from pea buds, which do not produce this globulin *in vivo*, failed to lead to its synthesis *in vitro* unless the histone had first been removed from the DNA. Barr and Butler (1963) made similar studies using DNA and histones from *Bos* and a protein-synthesizing system from *Bacillus megaterium*. The histone was fractionated by chromatography and other means, and appeared to consist of a limited number of characteristically different proteins, one, for instance, being rich in lysine and another in arginine. Barr and Butler found that complexes of the DNA with each histone fraction had a much reduced ability to promote RNA synthesis compared with DNA without histones. Although each fraction formed only 20–30% of the total histone, it reduced the activity of the DNA as primer to a much greater extent. Moreover, the artificial peptide poly-*l*-lysine was found to be an extremely effective inhibitor of RNA synthesis. Barr and Butler inferred that the effect of histone in inhibiting the priming activity of DNA was unspecific. They suggested that if there is only a limited number of histone types, there could be proteolytic enzymes or endopeptidases which act specifically on each type to remove them from the DNA. If the segments of histone of each type were of larger extent than the chromomeres, this might account for the length of the chromosome region affected by abnormal proximity to heterochromatin or, more specifically, to a controlling element within it.

Paul and Gilmour (1968) also obtained evidence that the effect of histones in masking the DNA is non-specific. They confirmed the discovery of Bonner and associates that organ-specific masking is preserved in isolated chromatin: the RNA of the thymus of the rabbit, *Oryctolagus cuniculus*, hybridised with 5–10% of rabbit DNA, and was synthesized *in vitro* when thymus chromatin was used as template; similar results were obtained with bone marrow RNA, part of which hybridized with a different fraction of the DNA. The specificity in the masking of the DNA appeared to reside, however, not in the histones but in a non-histone protein fraction. This was shown by the behaviour of the DNA as an RNA template when it was combined with each protein fraction separately. Thymus chromatin from *Bos*, when treated with dilute hydrochloric acid to remove the histones but leave other proteins associated with the DNA, had more template activity than the untreated chromatin but less than half that of pure DNA. Addition of the histones to the acid-treated chromatin restored the organ-specific template activity of the original chromatin. This is in contrast to the behaviour of the histones when added to the DNA alone, without the non-histone fraction: no RNA was then synthesized, evidently because the histones masked the DNA completely. Paul and Gilmour concluded that non-histone proteins are responsible for the unmasking of the genes which function in a particular tissue and that these proteins also prevent the non-specific masking of these genes by histones. It may be significant, as they point out,

that the repressor molecules isolated from bacteria and shown to bind to a specific nucleotide sequence in DNA are acidic proteins (§ 15.2), such as would be included in the non-histone fraction in their experiments, if similar proteins occurred in mammalian chromosomes.

Hamilton (1968) and Arnott *et al.* (1968) suggested that for transcription DNA may have to change its form so that the bases are tilted 20° to the helix axis, similar to double-helical RNA (§ 14.15); these two forms of DNA were recognised *in vitro* from X-ray crystallography by Franklin and Gosling (1953). Failure of base-tilting might thus provide a mechanism of gene inactivation.

§ 18.11 The cytoplasm in differentiation

In spite of these diverse pieces of evidence, which point to differential gene activity in the chromosomes as the basis of differentiation, there are cogent reasons for believing that such action forms only one aspect of the process. Thus, Spencer and Harris (1964), working with *Acetabularia crenulata*, found that synthesis of enzymes took place in enucleated cells during regeneration of the cap, and moreover that synthesis of one of the enzymes was specifically associated with the formation of the cap. The whole process of regeneration took 6–8 weeks. It was evident that enzyme synthesis can be regulated over long periods in the absence of the nucleus. A number of other observations support the belief that wholly cytoplasmic control of enzyme synthesis is of widespread occurrence in chromosomal organisms. Thus, Reich *et al.* (1962) showed that actinomycin *D* inhibited the formation of RNA in fibroblasts of *Mus* in tissue culture, but that protein synthesis, revealed by incorporation of tritiated leucine, continued for many hours, and Sokoloff *et al.* (1964) found that the addition of thyroxine to cell-free preparations of the liver of *Rattus* stimulated the incorporation of amino-acids into protein in the absence of any synthesis of RNA. Actinomycin *D* or deoxyribonuclease were used to prevent RNA synthesis.

That differential activity at the level of transcription cannot form more than a part of the mechanism of differentiation is also evident from study of the development of unicellular (or acellular) organisms such as *Acetabularia* or *Paramecium*, and from the embryology of multicellular organisms. From such studies it is apparent that differentiation occurs within cells as well as between one cell and another. Intracellular differentiation implies a spatial organization within the cell, and this presumably arises through interaction between molecules in the cytoplasm and physical or chemical factors in the external environment. In multicellular structures, this external environment would include other cells or tissues of the organism. There is little evidence, at present, about the means by which the spatial organization within a cell is determined, but the activity of an enzyme is known sometimes to be affected by genes other than its structural gene. The necessity for enzyme aggregates in the *arom* and *tryp* pathways in *Neurospora crassa* was discussed in § 15.10. Woodward and Munkres (1966) found that the activity of malate

dehydrogenase in *Neurospora* is affected by mutations of the structural protein of the mitochondrion, in association with which the enzyme functions. The mitochondrial protein is coded by a cytoplasmically-inherited gene (see below), while the structural genes for the two polypeptides of the enzyme are each in a different chromosome. Ganschow and Paigen (1967) identified a gene in the mouse, *Mus musculus*, mutation of which caused the enzyme glucuronidase to be absent from the ergastoplasm of liver cells, though still present in the lysosomes. This gene was not linked to the structural gene for the enzyme.

Differentiation within a cell includes the differentiation of the organelles which it contains. Clues as to how such differentiation may take place are provided by the development of virus *T*4 of *Escherichia coli*. Epstein *et al.* (1964) discovered two classes of conditional lethal mutants in this virus: temperature-sensitive mutants which formed plaques at 25°C but not at 42°C, and ambivalent (amber) mutants which formed plaques on a mutant (amber suppressor) strain of the host but not on the wild-type host (see § 13.8 and § 14.17). Both classes of mutations can occur in numerous genes, and have enabled the map positions and function of the genes to be established. In this way 77 genes, possibly representing about $\frac{3}{4}$ of the total number of genes in the *T*4 chromoneme, have been mapped (Fig. 18.3). Epstein *et al.* found that mutants which were defective in DNA synthesis failed to show synthesis and assembly either of the head or the tail components. It thus appeared that the mutants affecting DNA synthesis also prevented development proceeding past an early stage. This functional interaction defined what they called early- and late-acting genes, which (with a few exceptions) were also grouped together physically in the chromoneme (Fig. 18.3). A further point of great interest was that it appeared unlikely that there were more than 20 different proteins in the virus and so Epstein *et al.* imagined that many of the genes were 'morphogenetic' in function, that is, were involved in the assembly of the components rather than in their synthesis.

Study of the *in vitro* assembly of the virus particles by Edgar and Lielausis (1968) showed that there were three separate pathways concerned, respectively, with the head, the tail and the tail-fibres (Fig. 11.1). Within each pathway gene products interact in a fixed sequence. There is a tendency for genes concerned with each pathway to be clustered together on the linkage map (Fig. 18.3). King (1968) studied the formation of the tail of the virus and showed that the base-plate is completed first (*t*1 genes in Fig. 18.3) and then the core is formed on the base-plate (*t*2), followed by the sheath (*t*3–5). Unlike these steps, Edgar and Lielausis found that completed heads and tails combined spontaneously without gene control. The tail-fibres, products of the third independent assembly-line, were attached to the tail after it had combined with the head. These studies have not yet revealed, however, how many of the genes code for minor sub-units of the structure—nuts and bolts, as it were—and how many for enzymes which catalyse the joining of different parts, that is, act as temporary scaffolding.

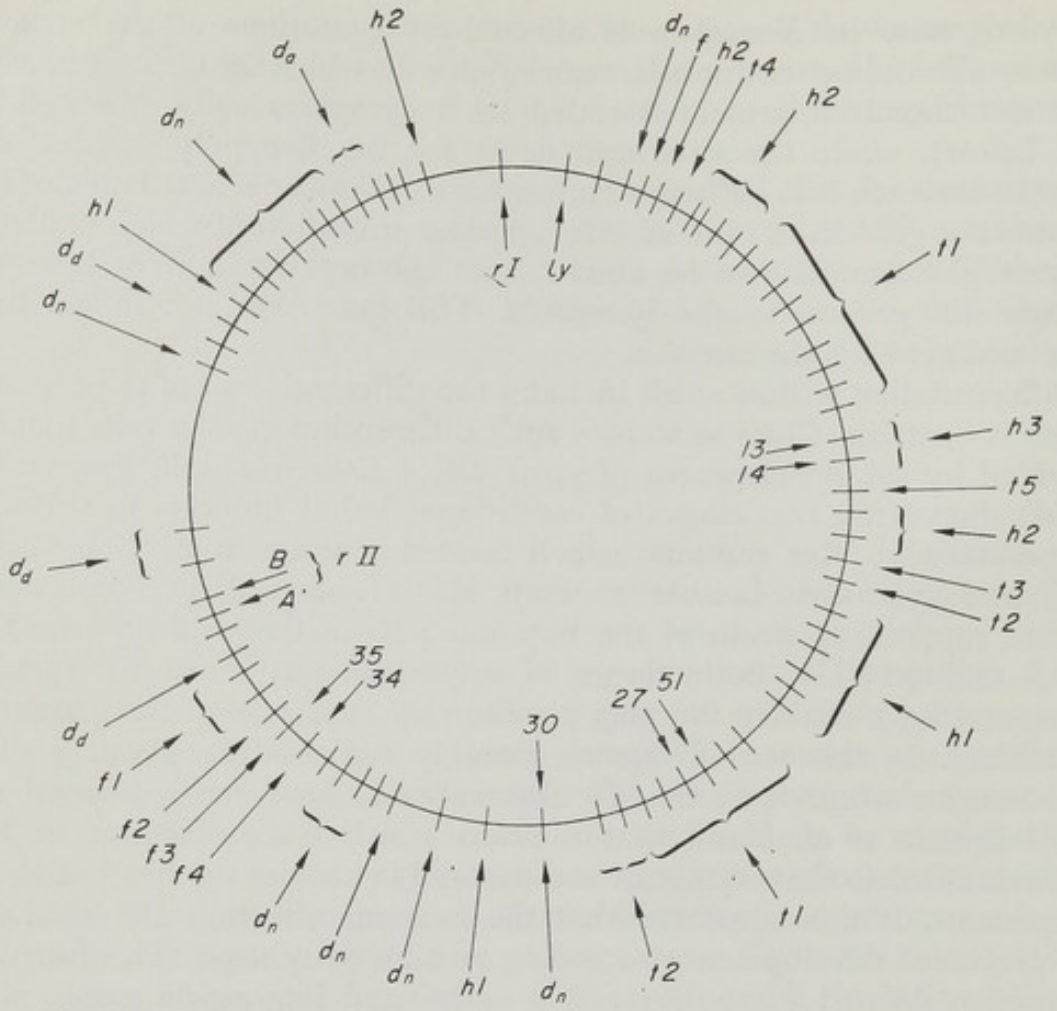


FIGURE 18.3 The diagram shows a genetic map of virus *T*4 based on the work of Edgar, Epstein and associates (see King, 1968). The virus has a linear, not circular, DNA molecule, but the map is circular because individual virus particles contain circular permutations of the nucleotide sequence, with some duplication of sequence at each end (see § 16.4). Symbols outside the circle indicate the function of the gene: *d*, early-acting genes—relating to DNA synthesis: d_a, DNA synthesis arrested after a short time; d_d, delay in starting DNA synthesis; d_n, no DNA synthesis (DNA negative); *f*, *h*, *t*, late-acting genes—*f*, fibres; *h*, head; *t*, tail. (See Fig. 11.1). Numerals associated with these letters indicate the sequence, as far as this is known, in which the products of these genes interact with one another: *f*1–3, formation of tail-fibre segments; *f*4, union of segments; *h*1, formation of protein coat of head; *h*2, formation of active head; *h*3, completion of head; *t*1, formation of active base-plate; *t*2, formation of core on base-plate; *t*3–5, formation and stabilization of sheath. Symbols inside the circle identify particular genes mentioned in the text: *ly*, gene for lysozyme.

The differentiation of cytoplasmic organelles in chromosomal organisms may perhaps follow a similar pattern to the assembly of *T*4, with genes for the formation of the essential components, and morphogenetic genes, giving products which interact in a fixed sequence.

It is inferred from Spencer and Harris' results described above that messenger RNA can persist for long periods in the cytoplasm of *Acetabularia*, and such persistence could also account for the examples of delayed Mendelian inheritance described in Chapter 5. On the other hand, the occurrence of cytoplasmic inheritance, also discussed in Chapter 5, indicates that some features of the cytoplasm are self-replicating rather than merely persistent. Many RNA viruses are known to be self-replicating. August, Cooper, Shapiro and Zinder (1964) demonstrated the existence of an RNA polymerase which is induced in *Escherichia coli* after infection by the RNA virus *f*2, and which uses the triphosphates of the 4 ribonucleosides as substrate, and leads to the production of replicas of the viral RNA without involving DNA (see Weissmann and Ochoa, 1967, for a review of phage RNA replication). So one possible explanation of cytoplasmic inheritance would be to postulate an RNA genetic system in the cytoplasm supplementary to the DNA system in the nucleus. There appears to be no evidence for the existence of such a system. However, it has recently been discovered that DNA occurs in the chloroplasts and mitochondria.

Plastids contain not less than 0·1 fg (1 femtogram = 10^{-15} g) of DNA or enough to code for 100 genes of 1000 nucleotide-pairs,* and mitochondria not less than 0·02 fg or 20 genes, and for each organelle the DNA has a characteristic base composition (see review by Granick and Gibor, 1967). Using the Kleinschmidt method, Sinclair and Stevens (1966) extracted the DNA from the mitochondria of *Mus* and found from electron micrographs that it was circular with a circumference of 5 μ, or the equivalent of 15 genes. Moreover, there is evidence, at least for plastids, that their DNA is unlikely to be derived wholly from the nucleus: Richards (1967) found that only about 15% of the chloroplast DNA of *Euglena gracilis* would hybridize with the nuclear DNA. Plastids and mitochondria are known to be self-replicating structures, and their DNA is known to replicate within the organelle, and to do so independently of the replication of the nuclear DNA (see Granick and Gibor, 1967, and Kirschner *et al.*, 1968).

In support of the idea that plastids and mitochondria are at least partially autonomous structures with their own genetic material, there is evidence that their DNA codes for specific RNAs and proteins associated with these organelles. As pointed out in § 14.5, plastids and mitochondria have smaller ribosomes than the rest of the cell. Scott and Smillie (1967) showed that ribosomal RNA from the chloroplasts of *Euglena* will anneal with chloroplast DNA. Also, the synthesis of enzymes involved in photosynthesis occurs on *Euglena* chloroplast ribosomes (Smillie *et al.*, 1967).

It was shown in Chapter 5 that many examples of cytoplasmic inheritance

* 1 fg $\equiv \frac{606{\cdot}2}{m} \times 10^6$ nucleotide-pairs, where m is the mean molecular weight of one nucleotide-pair of the DNA. This formula is derived from Avogadro's constant ($6{\cdot}062 \times 10^{23}$) which is the number of molecules in one gram molecule (for example, in 18 g of water). The molecular weights of nucleotide-pairs in the duplex polynucleotide are as follows: when the bases are adenine and thymine, 617·3; guanine and cytosine, 618·3; guanine and 5-methylcytosine, 632·3; guanine and 5-hydroxymethylcytosine, 648·3. It follows that 1 fg of DNA will usually be equivalent to about 980 genes of 1000 nucleotide-pairs.

are known, and that these are concerned predominantly with characters of the plastids and mitochondria. How far can cytoplasmic inheritance be attributed to genes in the DNA of these organelles? Woodward and Munkres (1966) discovered that two cytoplasmically-inherited respiratory-deficient mutants of *Neurospora crassa* were defective in the structural protein of the mitochondrion. One of the mutants had one less tryptophan and one more cysteine per molecule of the protein, a change which would be accounted for by a single base substitution (see Table 14.7). There is no evidence where in the cytoplasm this gene is situated, but it is natural to attribute it to the mitochondrial DNA. Mounolou *et al.* (1966) found that the mitochondrial DNA of cytoplasmic petite mutants of *Saccharomyces cerevisiae* (§ 5.15) was of abnormal density. Loss of the plastids of *Euglena* (§ 5.14) can be induced by irradiation of the cytoplasm, but not the nucleus, with ultraviolet light. A wavelength of 260 mμ gave the maximum effect, which could be reduced by exposure to visible light (see Granick and Gibor, 1967). Sensitivity to ultraviolet light of this wavelength, and photoreactivation, are characteristics of DNA. Similarly, Sager and Ramanis (1967) found that the behaviour of the cytoplasmic streptomycin and acetate mutants of *Chlamydomonas reinhardi* (see § 5.14) showed ultraviolet-sensitivity and photoreactivation. Moreover, they had found (Sager and Ramanis, 1965) that allelic mutants at the acetate locus showed linkage and recombination, and they recovered reciprocal recombinants in the same clone. Linkage and recombination were also found at the streptomycin locus. Sager and Ramanis argued that these results require a process of close intermolecular pairing and exchange such as is known only in nucleic acid. They concluded that their results supported the hypothesis that the cytoplasmic genes are composed of nucleic acid. Although not conclusively established, it thus seems probable that many of the known examples of cytoplasmic inheritance are caused by genes in the DNA of plastids and mitochondria. Both of these organelles evidently contain a chromoneme analogous to that of bacteria and viruses. Indeed, plastids and mitochondria may have originated from chromonemal organisms by some form of symbiosis, since their ribosomes are of chromonemal type.

It has long been realised that cytoplasmic inheritance does not necessarily imply the existence of self-perpetuating particles such as DNA or RNA might provide. Cytoplasmic mutant phenotypes might result from alteration of a self-perpetuating metabolic system from one steady state to another. This is exemplified by Monod and Jacob's hypothetical system described in § 18.6 where each of two alternative reaction-chains is inhibited by the end-product of the other. It would be necessary, in addition, to postulate that each chain contained features which made it self-perpetuating when once established. However, on present evidence the steps in the reaction-chains would be expected to depend on nuclear gene activity. An example of a condition that may be caused by cytoplasmic inheritance of this metabolic kind is the disease of sheep, *Ovis aries*, called scrapie. This disease develops slowly in the nervous system and was thought to be of viral origin until the causative agent was found to be insensitive to ultraviolet light and other treatments to which

nucleic acids are vulnerable. Pattison and Jones (1968) detected the scrapie agent in normal mice, *Mus musculus*, and suggested that the disease may be caused, not by self-replication of an infecting particle, but by unmasking of some cell constituent which is normally inhibited. Other diseases of the nervous system, such as multiple sclerosis and kuru in man, may have a similar cause.

It seems possible that an appreciable part of the cytoplasmic organization of a cell at any particular time may be a reflection of the previous nuclear activity. This is supported by studies of the development of, for example, echinoderms and *Acetabularia* (see Chapter 5). On the other hand, the occurrence of cytoplasmic inheritance demonstrates that the organisation cannot all be so explained, and that parts of the cytoplasm constitute self-perpetuating particles or reaction-chains. Until more is known biochemically about the nature of cytoplasmic inheritance, and about how species differ from one another cytoplasmically, the relative importance of Mendelian and non-Mendelian heredity cannot be judged with any precision, and likewise of nucleus and cytoplasm in the control of differentiation.

§ 18.12 The environment and gene activity

An important aspect of differentiation is the influence of the external environment, which can frequently modify profoundly the development of an organism. This is particularly evident in plants, where individuals growing in different habitats may differ greatly and yet when cultivated under similar conditions appear alike. An example of environmental influence is provided by sun red *Zea mays*, which has the genotype *A B pl pl*, that is, with the dominant genes *A* (anthocyanin pigment) and *B* (booster of 'plant colour'), and homozygous for the recessive gene *pl* (plant colour). In sun red maize, anthocyanin is formed only in those parts of the plant which are exposed to sunlight. Another example of the influence of the environment is provided by the number of facets to the eye in Bar-eyed *Drosophila melanogaster*. As indicated in § 9.10, the facet number is greatly influenced by the temperature during development: there are 4 or 5 times as many facets at 15°C than at 30°C. In general, to give precision to an account of the effect of a particular gene on an organism, the genotype and the environmental conditions must be specified. Many genes show *incomplete penetrance*, that is to say, they are not expressed in some individuals. Incomplete penetrance reflects variability of genotype or environment. If a gene acts at an early stage of development, it may affect many different characters of the organism, but the various parts may differ in their sensitivity to modification by other genetic factors and by the external environment. This gives rise to *variable expressivity* of the gene, that is, variation in the way in which a gene is expressed in different individuals. The plants of *Antirrhinum majus* found by Darwin to be intermediate in appearance between the normal and peloric forms (see Fig. 2.4) are likely to have arisen in this way.

How the environment influences gene activity is not understood. However, it appears significant that the variegated phenotypes associated both with

position effect and with mutable genes show great variability of expression depending on environmental factors. Harrison and Fincham (1964) have shown that the frequency of mutation to a magenta colour in the stems and petals of *A. majus*, which occurs with high frequency in strains of a particular genotype, is much influenced by the temperature when the tissue in question is developing. Many similar instances are known of sensitivity of mutable genes to the temperature at the stage of development when the gene is acting.

§ 18.13 Conclusions

From the experimental results described in this chapter the following tentative conclusions may be drawn.

The functional unit of the chromosome is the chromomere, which is a linear series of copies of a structural gene within one DNA molecule. Control of its activity seems to be possible in at least four ways. The primary control may involve a particular chemical, such as the end-product of a preceding step in a biosynthetic pathway or morphological assembly-line, interacting with an acidic protein which can recognize a specific nucleotide sequence in the DNA of the chromomere (or interchromomeric region alongside it). This protein would be coded by a regulatory gene.

A second control mechanism can cause the inhibition of gene activity in a segment of a chromosome or a whole chromosome as a result of the activation of a controlling element situated in heterochromatin. Such activation can lead to euchromatin becoming heterochromatic for a certain distance along the chromosome from the controlling element. Heterochromatin lacks chromomeres, as though the masking of their activity also masked their morphology. Histones seem to be a likely candidate for the molecules which cause this spreading effect, since they are believed to mask gene activity in a non-specific way.

Thirdly, controlling elements can cause frequent mutation in specific unlinked genes, possibly acting by way of episomes. The episome would be expected to be a circular DNA molecule. The mutability, which may be an abnormal feature, could arise from differences in nucleotide sequence between gene and episome. Mutability unrelated to a controlling element could arise from a failure of the matching process by which the slaves would have been rendered identical to the master copy of the gene.

A fourth control mechanism may occur at the translation of messenger into polypeptide. Ribosomes, transfer RNAs or the conformation of the messenger might provide the control. Ribosomal RNA and transfer RNAs are themselves the products of special genes which determine RNA nucleotide sequences for their own sake.

Each of these control mechanisms involves a different molecule (acidic protein, histone, DNA, RNA) and a different process. Genetic and environmental factors would be expected to influence all of them, but not in a uniform way in view of their diversity. A further complication is the probable existence of subsidiary hereditary systems in plastids and mitochondria, most probably with a chromonemal type of organization.

The basic unit of the chromosome from the point of view of its replication also seems to be the chromomere. A control mechanism evidently exists which determines the sequence in which replication is initiated in the various replicons. That heterochromatin replicates late suggests that whatever masks the chromomeres morphologically and functionally in heterochromatic segments also temporarily masks them from the signals which trigger the replication of the euchromatic replicons.

The basic unit of the chromosome from the point of view of recombination also seems to be the chromomere. Here, too, there is evidence for specific control in each chromomere. Moreover, crossing-over is normally absent from heterochromatin. This was shown, for example, by Baker (1958) with *Drosophila*. Woollam and Ford (1964) found that the chromosome material lateral to the synapton was much denser in heterochromatic than in euchromatic regions of *Mus* chromosomes. Assuming that crossing-over occurs in the synapton, there is evidently much less opportunity for crossing-over in heterochromatin than euchromatin. There is a possibility that regulatory genes and controlling elements (which occur in heterochromatin) recombine by means of episomes and not by crossing-over (§ 16.7).

The similarity in the organization of all three primary functions of the hereditary material—its specific action, its replication and its recombination—is remarkable. All seem to depend on the chromomere as the basic unit, to be under specific control at the level of the individual chromomere, and to be subject to delay or inactivation when the chromomere becomes heterochromatic. The existence of a common unit for all three functions raises the question whether they act through a common mechanism: a transcriptase, a replicase and a recombinase might each be capable of functioning in every unit, but be active only when combined with the key—a specific activator or derepressor—for unlocking that unit.

Appendix 1. Organisms mentioned in the text

(In order to make the classification more meaningful, a few species and phyla have been included which are not referred to in the text.)

I. CHROMONEMAL KINGDOM

The term 'chromoneme' is used for 'bacterial chromosome' (see § 11.1). Chromonemal organisms differ from chromosomal in having no basic proteins (histones or protamines) associated with their DNA, no mitotic apparatus, no nuclear membrane, no mitochondria, and no plastids and in having ribosomes with a sedimentation coefficient of 70*S* instead of 80*S*. The cell-walls have a distinctive composition containing mucopeptides, and the flagella, where present, are of a different construction from those of chromosomal organisms. Lysine synthesis is by a pathway involving diaminopimelic acid (Vogel, 1965). For a detailed account of the characteristic features of chromonemal organisms, see Stanier (1964). The relationship between bacteria and blue-green algae has been discussed by Echlin and Morris (1965).

A. BACTERIA

Photosynthesis absent, or, if present, without evolution of oxygen.

(i) EUBACTERIALES

a Pseudomonadaceae
Pseudomonas aeruginosa

b Azotobacteriaceae
Azotobacter vinelandii

c Micrococcaceae
Micrococcus pyogenes

d Parvobacteriaceae
Haemophilus influenzae

e Lactobacteriaceae
Diplococcus pneumoniae (Pneumococcus)
Streptococcus faecalis

f Achromobacteriaceae

g Enterobacteriaceae
Aerobacter aerogenes
Escherichia coli (Colon bacillus)
Salmonella typhimurium (Mouse typhoid bacillus)

h Bacillaceae
Bacillus cereus
—megaterium
—subtilis

(ii) ACTINOMYCETALES
a Mycobacteriaceae
Mycobacterium phlei
—tuberculosis avium (Avian tubercle bacillus)
b Streptomycetaceae
Streptomyces coelicolor
—griseus

B. CYANOPHYTA
(Myxophyta, Blue-green Algae)
Photosynthesis with evolution of oxygen
Phormidium luridum

II. CHROMOSOMAL KINGDOM

The classification of fungi with a motile stage follows Sparrow (1959), whose division of them into the truly fungal Chytridiomycetes and the algal Phycomycetes has received strong support from the remarkable discovery by Vogel (1965) that the true fungi have a different pathway for lysine synthesis from other plants, and so have probably evolved from animals. *Euglena* has the lysine pathway of fungi and is therefore presumably of fungal origin, having perhaps become photosynthetic secondarily by symbiosis with a chloroplast from another organism.

A. PLANT SUBKINGDOM

Lysine synthesis by the diaminopimelic acid pathway.

(i) CHLOROPHYTA (Green Algae)
a Volvocales
1 Green forms
Chlamydomonas reinhardi
2 Colourless forms
Polytoma sp.
b Siphonales
Acetabularia crenulata
—mediterranea

(ii) XANTHOPHYTA
a Xanthophyceae (Yellow-green Algae)
Vaucheria sp.
b Phycomycetes (Zoospores biflagellate or anteriorly uniflagellate)
Pythium sp.

(iii) PHAEOPHYTA (Brown Seaweeds)

(iv) RHODOPHYTA (Red Seaweeds)
(v) BACILLARIOPHYTA (Diatoms)
(vi) BRYOPHYTA
a Musci (Mosses)
b Hepaticae (Liverworts)
Pellia endiviifolia
Sphaerocarpos donnellii
(vii) PTERIDOPHYTA (Ferns, etc.)
Ophioglossum petiolaris (Adder's-tongue Fern)
(viii) GYMNOSPERMAE (Conifers, Cycads, etc.)
(ix) ANGIOSPERMAE (Flowering Plants)
a Monocotyledones
1 Gramineae (Grasses)
Avena sativa (Oats)
Hordeum vulgare (Barley)
Oryza sativa (Rice)
Triticum aestivum (Wheat)
Zea mays (Maize)
2 Commelinaceae
Tradescantia paludosa
3 Juncaceae (Rushes)
Luzula sp. (Wood-rush)
4 Liliaceae (Lilies, etc.)
Allium cepa (Onion)
—cernuum
—fistulosum
Aloe purpurascens
Bellevalia romana
Disporum sessile
Endymion nonscriptus (Bluebell)
Fritillaria sp.
Hyacinthus orientalis (Hyacinth)
Ipheion uniflorum
Lilium formosanum
—henryi
—japonicum
—longiflorum
—maximowiczii
—regale
Paris verticillata
Scilla scilloides
—sibirica
Trillium erectum
Tulipa sp. (Tulip)
5 Amaryllidaceae
Clivia cytranthiflora
Haemanthus katherinae
6 Iridaceae
Crocus balansae

7 Orchidaceae (Orchids)

b Dicotyledones

1 Urticaceae
Urtica pilulifera (Roman Nettle)

2 Nyctaginaceae
Mirabilis jalapa (Marvel of Peru)

3 Caryophyllaceae
Agrostemma githago (Corn Cockle)
Silene alba (White Campion)
—dioica (Red Campion)

4 Ranunculaceae
Anemone spp.
Helleborus foetidus

5 Papaveraceae
Chelidonium majus (Greater Celandine)
Papaver somniferum (Opium Poppy)

6 Cruciferae
Lunaria annua (Honesty)

7 Leguminosae
Lathyrus odoratus (Sweet Pea)
Phaseolus vulgaris (Dwarf Bean)
Pisum sativum (Edible Pea)
Trifolium pratense (Red Clover)
Vicia faba (Broad Bean)

8 Geraniaceae
Pelargonium zonale (Geranium)

9 Linaceae
Linum usitatissimum (Flax)

10 Violaceae
Viola cornuta

11 Caricaceae
Carica papaya (Papaw)

12 Onagraceae
Epilobium hirsutum (Hairy Willow-herb)
—luteum
Oenothera blandina
—hookeri
—lamarckiana (Evening Primrose)
—muricata

13 Primulaceae
Primula sinensis

14 Labiatae
Salvia horminum

15 Solanaceae
Datura tatula
—stramonium (Thorn Apple)
Hyoscyamus niger (Henbane)
Nicotiana tabacum (Tobacco)
Solanum nigrum (Black Nightshade)

16 Scrophulariaceae
Antirrhinum glutinosum
—majus (Snapdragon)
Veronica longifolia
17 Plantaginaceae
Plantago lanceolata (Ribwort Plantain)
18 Compositae
Aster tripolium (Sea Aster)
Chrysanthemum roxburghi
Coreopsis tinctoria
Haplopappus gracilis

B. FUNGAL SUBKINGDOM

Lysine synthesis by a pathway involving α-aminoadipic acid.

(i) CHYTRIDIOMYCETES (Zoospores posteriorly uniflagellate)
Allomyces sp.
(ii) EUGLENIDA
Euglena gracilis (including *Astasia longa*)
(iii) ZYGOMYCETES
Mucorales
Mucor sp.
(iv) ASCOMYCETES
a Endomycetales (Yeasts, etc.)
Saccharomyces cerevisiae
Torulopsis utilis*
b Eu-Ascomycetes
1 Plectomycetes
Aspergillus nidulans
*—niger**
*Penicillium notatum**
2 Discomycetes
Ascobolus immersus
Neottiella rutilans
3 Pyrenomycetes
Bombardia lunata
Neurospora crassa
—sitophila (Red Bread Mould)
Sordaria brevicollis
—fimicola
(v) BASIDIOMYCETES
a Uredinales (Rust Fungi)
Puccinia glumarum (Yellow Rust)
b Ustilaginales (Smut Fungi)
Ustilago maydis (Maize Smut)
c Hymenomycetes (Mushrooms and Toadstools)
Schizophyllum commune
d Gasteromycetes (Puffballs)

* Fungi Imperfecti: nuclear fusion and meiosis unknown.

C. ANIMAL SUBKINGDOM

Lysine not synthesized.

(i) PROTOZOA

a Mycetozoa (Slime Moulds)

1 Myxomycetales (Plasmodial Slime Moulds)

Physarum sp.

2 Acrasiales (Cellular Slime Moulds)

Dictyostelium sp.

b Sarcodina

Amoeba sp.

c Flagellata (Zoomastigina)

Trypanosoma sp. (Sleeping sickness parasite)

d Sporozoa

Plasmodium sp. (Malarial parasite)

e Ciliata

Holotricha

Paramecium aurelia

(ii) PORIFERA (Sponges, etc.)

(iii) COELENTERATA (Jelly-fish, etc.)

(iv) PLATYHELMINTHES (Flatworms)

Mesostoma spp.

(v) NEMATODA (Threadworms)

Parascaris equorum (*Ascaris megalocephala*) (Horse Threadworm)

(vi) ANNELIDA (Worms)

(vii) ARTHROPODA

a Crustacea (Shrimps, Crabs, etc.)

Gammarus spp.

Procambarus clarkii (Crayfish)

b Myriapoda (Centipedes)

c Insecta (Insects)

1 Orthoptera (Grasshoppers)

Acrida lata

Arphia simplex

Brachystola magna

Calliptamus palaestinensis

Chorthippus brunneus

—parallelus

Cyrtacanthacris tartarica (Locust)

Gryllotalpa hexadactyla (Mole Cricket)

Melanoplus differentialis

—femur-rubrum

Phrynotettix tschivavensis (*P. magnus*)

Romalea microptera

Schistocerca gregaria (Locust)

Stauroderus bicolor

Trimerotropis maritima

2 Hemiptera (Bugs)
Euschistus variolarius
Lygaeus turcicus
Oncopeltus fasciatus
Protenor belfragei
Pyrrhocoris apterus
Thyanta spp.

3 Lepidoptera (Butterflies, Moths)
Abraxas grossulariata (Magpie Moth)
Ephestia kuhniella

4 Coleoptera (Beetles)
Tenebrio molitor (Meal worm)

5 Hymenoptera (Ants, Bees, Wasps)
Apis mellifica (Honey Bee)

6 Diptera (Flies)
Chironomus pallidivittatus
—tentans
—thummi subsp. *piger*
— —subsp. *thummi*
Drosophila hydei
—melanogaster (Fruit Fly)
—pseudo-obscura
—subobscura

d Arachnida (Scorpions, Spiders)

(viii) MOLLUSCA
a Lamellibranchiata (Oysters, etc.)
b Gasteropoda (Snails, Slugs)
Helix hortensis (Garden Snail)
—nemoralis
Limnaea peregra (Freshwater Snail)
c Cephalopoda (Octopus, etc.)

(ix) ECHINODERMATA (Sea Urchins, etc.)
Lytechinus variegatus
Paracentrotus lividus
Psammechinus microtuberculatus
Sphaerechinus granularis
Strongylocentrotus purpuratus

(x) CHORDATA (Vertebrates, etc.)
a Pisces (Fish)
1 Teleostei
Alosa sp. (Shad)
Salmo spp. (Salmon, Trout)
2 Elasmobranchii
Carcharias spp. (Shark)

3 Dipnoi
Protopterus sp. (Lung-fish)

b Amphibia (Amphibians)
1 Anura (Frogs, Toads)
Bufo bufo (European Toad)
—viridis (Green Toad)
Rana pipiens (American Frog)
—temporaria (European Frog)
Xenopus laevis (Clawed Toad)
2 Urodela (Newts, Salamanders)
Ambystoma mexicanum (Axolotl)
Batrachoseps attenuatus (Slender Salamander)
Salamandra maculosa (Spotted Salamander)
Triturus cristatus (European Crested Newt)
—pyrrhogaster
—viridescens (American Newt)

c Reptilia (Reptiles)
Crotalus adamanteus (Diamond-back Rattlesnake)

d Aves (Birds)
Gallus domesticus (Domestic Fowl)

e Mammalia (Mammals)
1 Marsupialia
Potorous tridactylus (Rat Kangaroo)
2 Lagomorpha (Rabbits, Hares)
Oryctolagus cuniculus (Rabbit)
3 Rodentia (Rodents)
Cricetulus griseus (Chinese Hamster)
Mesocricetus auratus (Golden Hamster)
Mus musculus (Mouse)
Rattus norvegicus (Rat)
4 Cetacea (Whales)
Balaenoptera borealis (Sei Whale)
5 Carnivora (Carnivores)
Canis familiaris (Dog)
Felis catus (Cat)
6 Perissodactyla (Odd-toed Ungulates)
Equus caballus (Horse)
7 Artiodactyla (Even-toed Ungulates)
Bos taurus (Domestic Cattle)
Ovis aries (Sheep)
Sus scrofa (Pig)
8 Primates
Homo sapiens (Man)
Macacus mulatta (Rhesus Monkey)

III. VIRUSES

For the classification of viruses, see Lwoff and Tournier (1966).

A. DEOXYVIRA (DNA Viruses)

(i) DEOXYHELICA (Nucleocapsid with helical symmetry) *Poxvirus variolae* (Small-pox virus)

(ii) DEOXYCUBICA (Nucleocapsid with cubical symmetry)
Microvirus monocatena (ϕX174)

(iii) DEOXYBINALA (With head and tail)
Phagovirus (*Coli*) *T secundus* (T2)
The other T phages and λ, 434, ϕ80 and P22 belong to this class.

B. RIBOVIRA (RNA Viruses)

(i) RIBOHELICA (Nucleocapsid with helical symmetry)
Protovirus tabaci (Tobacco mosaic virus)
Ribgrass virus belongs to this genus.

(ii) RIBOCUBICA (Nucleocapsid with cubical symmetry)

a Napoviridae
Androphagovirus bacterii (RNA phage)
R17 and f2 belong to this genus.

b Reoviridae
Reovirus (*Mammalis*) *primus* (Reovirus I)

Appendix 2. Glossary of genetical terms used in the text

Acentric Applied to a chromatid or a chromosome when it lacks a centromere. This condition may arise in an inversion heterozygote as a result of crossing-over between a normal and an inverted segment that does not include the centromere.

Allele (allelomorph) The term coined by Bateson and Saunders (1902) for characters which are alternative to one another in Mendelian inheritance (Gk. *allelon*, one another; *morphe*, form). The term is also applied to the wild-type and mutant forms of a gene responsible for such a character-difference.

Anaphase Strasburger (1884) originally introduced this term for the stage of nuclear division when the contents of the nuclei were going back (Gk. *ana*) to their normal appearance, but from about 1905 he used the term in the now universally adopted sense of the stage of mitosis or of meiosis I or II when the daughter-chromosomes (or homologous chromosomes at meiosis I) move towards opposite poles of the spindle.

Anticodon The triplet of nucleotides in transfer RNA which associates by complementary base pairing with a specific triplet (codon) in messenger RNA during protein synthesis.

Autosomes A term coined by Montgomery (1906) for the chromosomes other than the *X*- and *Y*-chromosomes (which he called allosomes) by which the sexes differ.

Back-cross A cross between a parental strain and its progeny from a previous cross.

Centromere The term introduced by Darlington (1936*a*) for the point on a chromosome which reacts to the spindle at nuclear division, and to which a spindle-fibre appears to be attached at the times when the spindle is evident (metaphase and anaphase) (Gk. *centron*, centre; *meros*, part). The chromosome is usually constricted and often bent at the centromere. In a few organisms the centromeric properties are distributed over the entire length of the chromosome (diffuse centromere).

Chiasma The term which Janssens (1909) introduced for the nodes (Gk. *chiasma*, cross) where the individual chromosomes making up each pair remain in contact during the diplotene and diakinetic stages of prophase I and during metaphase I of meiosis. It is now established that a chiasma arises as a consequence of crossing-over. In some organisms the chiasmata move towards the ends of the chromosome arms during late

diplotene and diakinesis (terminalization of chiasmata). The position of the chiasma will then no longer correspond to that of the cross-over. The expression 'terminal chiasma' was coined by Newton and Darlington (1929) for the end-to-end association of homologous chromosome arms as a result of chiasma movement to the end.

Chromatid The term which McClung (1900) proposed for each of the four threads making up a chromosome-pair at meiosis (Gk. *chroma*, colour; for the derivation of *id*, see Diploid). The use of the term was subsequently extended to mitosis, and is now applied to the individual daughter-chromosomes into which each chromosome is divided in all nuclear divisions. The term chromatid is used so long as the daughter centromeres remain in contact with one another. As soon as they separate (anaphase of mitosis and anaphase II of meiosis), the expression daughter-chromosome is substituted for chromatid.

Chromomere The term applied by Wilson (1896) to each of the serially aligned granules of a chromosome, best seen when the thread is relatively elongated as in early prophase I of meiosis (Gk. *chroma*, colour; *meros*, part).

Chromoneme The term used in this book for the DNA thread of bacteria and their viruses (see § 11.1 and Appendix 1) (Gk. *chroma*, colour; *nema*, thread). It is appropriate also to use it for the DNA of plastids and mitochondria.

Chromosome The term proposed by Waldeyer (1888) for the individual threads within the cell nucleus (Gk. *chroma*, colour; *soma*, body). The expression lampbrush chromosome describes the appearance of the chromosomes at the diplotene stage of meiosis in the oocytes of some amphibia, when the chromatids extend from each chromomere to form lateral loops. Wilson (1909) proposed that a chromosome which is represented twice (the normal diploid condition) in the nuclei of one sex, but is present only once per nucleus in the other sex, should be called an *X*-chromosome, while the dissimilar chromosome which may also be present as a single chromosome in the latter sex, but is absent from the sex with two *X*-chromosomes, he called a *Y*-chromosome. The normal chromosomes were called by Randolph (1928) *A*-chromosomes to distinguish them from supernumerary or *B*-chromosomes which occur in some individuals or populations of certain species, and are usually composed wholly of heterochromatin.

Cis The term proposed by Haldane (1941), by analogy with chemical isomerism, to describe a double heterozygote in coupling phase, that is, with the two dominant factors derived from one parent and the two recessives from the other. The term is used chiefly for closely linked mutants, to which it was first applied by Pontecorvo (1950). The cis configuration for two recessive mutants, 1 and 2, is $\frac{++}{1\ 2}$, where + stands for the wild-type, and the line separates the contributions from the two parents. (Cf. Trans.)

Cistron The term which Benzer (1957) introduced, derived from cis and

trans, for the functional unit of the hereditary material defined by the phenotype of the trans (repulsion) heterokaryon or heterozygote for two recessive mutations: if this phenotype is mutant, the mutations are said to be alleles and to belong to the same cistron; if the phenotype is normal (wild-type), the mutations are said to be non-allelic and to belong to different cistrons. Benzer's definition has since been modified to accommodate allelic complementation, and a cistron is now defined (Fincham 1959*a*) as a segment of the hereditary material within which pairs of mutations in the trans configuration are either deficient for a particular enzyme, or give rise to that enzyme in a structurally abnormal form. There is a good case for equating Fincham's definition of a cistron with the term gene.

Codon The term proposed by Crick (1963) for the sequence of nucleotides in DNA or RNA which is responsible for determining that a specific amino-acid shall be inserted into a polyptptide chain. There is more than one codon for most amino-acids, and it has now been established that the codons are triplets of nucleotides.

Complementation The process by which two recessive mutants can supply each other's deficiency, such that a heterokaryon or diploid derived from them and having the trans (repulsion) configuration is phenotypically normal (wild-type) or nearly so. Allelic complementation appears to be due to the formation of hybrid protein molecules.

Conversion The term proposed by Winkler (1930) for a process of interaction between alleles at meiosis. The term was re-introduced by Lindegren (1953) to account for aberrant ratios in the products of meiosis, apparently arising from such interaction. Whitehouse and Hastings (1965) have suggested that if gene conversion is due to the correction of mispairing of bases in DNA, it may give rise to reciprocal as well as non-reciprocal recombination.

Coupling The term introduced by Bateson, Saunders and Punnett (1905) for the condition (phase) of a heterozygote for two linked character-differences in which the two dominant factors were derived from one parent and the two recessive factors from the other. This condition may be represented as $\frac{A\ B}{a\ b}$, where A/a and B/b represent the two character-differences and the capital letters indicate the dominants. The horizontal line separates the contribution from each parent. (Cf. Repulsion.)

Crossing-over The term coined by Morgan and Cattell (1912) for the occurrence of new combinations of linked characters. With the acceptance of the chromosome theory, the term is also applied to the process of exchange between homologous chromosomes which gives rise to the new character-combinations.

Deficiency
Deletion } Loss of a segment of a chromosome.

Diakinesis The term coined by Häcker (1897) for the stage of late prophase I of meiosis when the chromosomes are well separated from one another (Gk. *kinesis*, movement; *dia*, apart). This stage is recognized by

the highly condensed condition of the chromosomes, the homologous pairs of which are held together by chiasmata. In some organisms, just before or during diakinesis the chiasmata move to the ends of the chromosome arms (terminalization of chiasmata). When there are interstitial chiasmata, the successive loops into which the chromosome-pair is divided are set in planes at right angles to one another.

Dicentric Applied to a chromatid or chromosome with two centromeres, such as arises in an inversion heterozygote as a result of crossing-over between a normal and an inverted segment that does not include the centromere.

Diploid With two homologous sets of chromosomes, such as arise at fertilization. The term was introduced by Strasburger (1905), and is derived from the Greek *diploos* meaning double, and *id*, which was Weismann's term for hypothetical structural units of the nucleus assumed to be represented by the chromomeres. The term *id* was derived from Nägeli's term idioplasm (Gk. *idion*, peculiar).

Diplotene The term proposed by von Winiwarter (1900), originally as an adjective, to describe the nuclei at a particular stage of prophase I of meiosis when the two chromosomes making up each homologous pair have separated from one another except at nodes (chiasmata) distributed along their length. The successive loops between the chiasmata all lie in one plane. (Gk. *diploos*, double; *taenia*, ribbon.)

Dominant The term which Mendel (1866) introduced for a character which is manifest in all the members of the first filial (F_1) generation from a cross between two pure-breeding strains differing in respect of this character, and which is evident in three quarters of the individuals of the second filial (F_2) generation. (Cf. Recessive.)

Duplication The repetition of a sequence of chromomeres or other chromosome segment, usually in linear juxtaposition to the original.

Episome The term introduced by Jacob and Wollman (1958*b*) for a particle which may at some time exist attached to and apparently integrated with the hereditary material of a cell, and which may at other times occur free from such attachment. When attached, episomes replicate along with the hereditary material, but when free they usually replicate independently of it and more rapidly. Examples of episomes are many bacterial viruses and the male sex factor in *Escherichia coli*. (Gk. *epi*, upon; *soma*, body.)

Euchromatin Parts of chromosomes showing the normal cycle of condensation and normal staining properties at nuclear divisions. (Gk. *eu*, true.) (Cf. Heterochromatin.)

Gene The term coined by Johannsen (1909) for the unit of heredity, that is, the hypothetical entity which determines the development of a particular character. The word gene was derived from De Vries' term pangen, itself a derivative of the word pangenesis which Darwin (1868) had coined. At one time it was assumed that the hereditary units defined by mutation, recombination, and function were the same, but with the discovery that

they are not, the term gene is now usually applied to the unit of function, that is, the cistron.

Genetics The term which Bateson (1907*a*) coined for the science of heredity and variation. (Gk. *genesis*, descent.)

Genotype The term proposed by Johannsen (1909) for the hereditary constitution of an individual, or of particular nuclei within its cells.

Haploid With a single set of chromosomes, such as occurs in gamete nuclei (Strasburger, 1905). (Gk. *haploos*, single; for the derivation of *id*, see Diploid.)

Heterochromatin The term proposed by Heitz (1928) for parts of chromosomes with an abnormal degree of contraction or of staining properties at nuclear divisions. (Gk. *heteros*, different; chromatin was Flemming's term for the material of which the chromosomes are composed.) (Cf. Euchromatin.)

Heterokaryon A multinucleate cell containing nuclei of more than one genotype, such as are of widespread occurrence in fungi (Gk. *heteros*, different; *karyon*, nut, nucleus).

Heterozygote The term coined by Bateson and Saunders (1902) for a zygote, or a diploid individual derived from it, which carries both members (Gk. *heteros*, different) of a pair of alleles. This might be as a result of fusion of dissimilar gametes, or from mutation.

Homokaryon A multinucleate cell containing nuclei of only one genotype. (Gk. *homos*, alike; *karyon*, nut, nucleus.)

Homozygote The term coined by Bateson and Saunders (1902) for a zygote, or a diploid individual derived from it, which carries only one member of a pair of alleles, that is, a zygote derived from the union of gametes identical (Gk. *homos*, alike) in respect of a particular gene.

Interchange An exchange of segments between non-homologous chromosomes. In an interchange heterozygote, the chromosomes contributed by one parent have the normal configuration and those contributed by the other parent have the interchange of segments.

Interference The term proposed by Muller (1916) for the interaction between crossovers such that the occurrence of one exchange between homologous chromosomes reduces the likelihood of another in its vicinity. Chromatid interference is the expression which Mather (1933*a*) introduced for a non-random distribution between the chromatids involved in successive crossovers.

Inversion The term introduced by Sturtevant (1926) for the reversal of the linear sequence of the genes in a segment of a chromosome. In an inversion heterozygote one of the chromosomes contributed by one parent has an inverted segment while the homologous chromosome contributed by the other parent has the normal gene sequence.

Leptotene The term proposed by von Winiwarter (1900), originally as an adjective, to describe the nuclei at the earliest stage of prophase I of meiosis, when the chromosomes first become visible as fine threads and have not yet associated in pairs. (Gk. *leptos*, thin; *taenia*, ribbon.)

Linkage An association in inheritance between characters such that the parental character-combinations appear among the progeny more often than the non-parental. Thus, if the parental combinations were *AB* and *ab*, where *A*/*a* and *B*/*b* are two pairs of contrasting characters, then linkage is shown if *AB* and *ab* are significantly more frequent than *Ab* and *aB* among the progeny.

Locus The position occupied by a gene in the chromosome. (Cf. Site.)

Meiosis The term coined by Farmer and Moore (1905) to describe the special nuclear divisions associated with the halving (Gk. *meion*, smaller) of the chromosome number in compensation for the doubling at fertilization. Two successive nuclear divisions occur, with no intervening chromosome replication. Grégoire (1904) proposed that the two divisions be denoted by the appropriate Roman numerals I and II. At the first division of meiosis, homologous chromosomes, already divided into chromatids, associate, undergo crossing-over at certain points along their length, and subsequently separate to opposite poles of the spindle. At the second division, their chromatids separate, leading to four haploid nuclei.

Metaphase Strasburger (1884) originally introduced this term for the stage of nuclear division after (Gk. *meta*) the chromosomes have divided into chromatids, but from about 1905, with the realization that the chromosomes are already double when nuclear division begins, he used the term in the now universally adopted sense of the stage of mitosis or of meiosis I or II when the chromosomes lie on the equatorial plate of the spindle.

Mispairing The presence in one nucleotide chain of a DNA molecule of a nucleotide which is not the complement of that at the corresponding position in the other chain.

Mitosis The term proposed by Flemming (1882) for the process of nuclear division, during which the chromosomes become evident as they shorten and thicken (prophase), and then take up position on the equatorial plate of the spindle (metaphase), to be followed by separation of the two daughter-chromosomes of which each is composed to opposite poles of the spindle (anaphase), and their gradual return to a slender elongated condition (telophase). (Gk. *mitos*, thread.)

Mutation The term which De Vries introduced into biological literature for an abrupt change of phenotype which is inherited. Mutation may be due to a molecular change within a gene, or to the loss or duplication of one or more entire genes.

Nucleolus The more or less spherical structure which occurs in association with a particular point (the nucleolar organizer) on a specific chromosome in the nucleus. At nuclear divisions, the nucleoli disappear in late prophase, are absent at metaphase and anaphase, and reappear during telophase.

Nucleus The term proposed by Brown (1833) for the more or less spherical structure which occurs in cells and stains deeply with basic dyes.

Operator The term introduced by Jacob, Perrin, Sanchez and Monod

(1960) for the site at one end of an operon where a repressor molecule binds to the DNA and thereby inhibits transcription. The term is appropriate also for the corresponding site when a gene does not form part of an operon.

Operon The term which Jacob, Perrin, Sanchez and Monod (1960) introduced for a group of closely-linked genes which appear to affect different steps in a single biosynthetic pathway and which appear to function as an integrated unit. With the discovery that the genes of an operon are transcribed in one messenger RNA molecule, an operon could be defined as an aggregate of genes with a single messenger.

Pachytene The term proposed by von Winiwarter (1900), originally as an adjective, to describe the nuclei at a particular stage of prophase I of meiosis when homologous chromosomes are associated throughout their length. (Gk. *pachys*, thick; *taenia*, ribon.)

Phenotype The term coined by Johannsen (1909) for the appearance (Gk. *phainein*, to appear) of an organism with respect to a particular character or group of characters, as a result of its genotype and its environment.

Polyploid Having three or more (Gk. *polys*, many) sets of homologous chromosomes. The term was introduced by Strasburger (1910) by analogy with his earlier terms haploid and diploid.

Polytene The term proposed by Koller (1935) to describe the multi-stranded condition (Gk. *polys*, many; *taenia*, ribbon) of the chromosomes found in certain specialized tissues such as the salivary glands of *Drosophila*.

Postmeiotic Segregation See Segregation.

Promoter The term introduced by Jacob, Ullman and Monod (1964) for a site at one end of an operon necessary for its expression, and now identified as the site where the synthesis of messenger RNA (transcription) is initiated (Ippen, Miller, Scaife and Beckwith, 1968). The term is appropriate also for the corresponding site when a gene does not form part of an operon.

Prophase Strasburger (1884) originally introduced this term for the early stage of nuclear division before (Gk. *pro*) the chromosomes divide into two chromatids, but from about 1905, with the realization that the chromosomes are double from the beginning of nuclear division, he used the term in the now universally adopted sense of the stage of mitosis or of meiosis I or II before breakdown of the nuclear membrane.

Recessive The term which Mendel (1866) proposed for a character which was not evident in the first filial generation (F_1) of a cross between two pure-breeding strains differing in respect of this character, and which re-appeared in one quarter of the second filial generation (F_2). (Cf. Dominant.)

Recombination The process by which new combinations of parental characters may arise in the progeny. Recombination may come about through random orientation of non-homologous chromosome-pairs on the meiotic spindles, from crossing-over between homologous chromosomes, from gene conversion, or by other means.

Replicon The term proposed by Jacob and Brenner (1963) for a unit of replication of the hereditary material, that is, a segment within which replication occurs in a unified way from a pre-determined origin in a fixed direction to a pre-determined end-point.

Repulsion The term introduced by Punnett and Bateson (1908) for the condition (phase) of a heterozygote for two linked character-differences in which the two dominant factors were derived one from each parent of the individual, and likewise with the two recessive factors. This condition may be represented as $\frac{A\ b}{a\ B}$, where A/a and B/b represent two pairs of alleles, and the capital letters represent the dominants. The horizontal line separates the contribution from each parent. (Cf. Coupling.)

Ribosome The term proposed by Roberts (1958) for the *ribo*nucleoprotein particles (Gk. *soma*, body) associated with protein synthesis.

Segregation The separation of allelic differences from one another. Segregation may occur at the first or second division of meiosis, or at the first mitosis after meiosis (postmeiotic segregation).

Semi-conservative The term proposed by Delbrück and Stent (1957) to describe the method of replication of DNA postulated by Watson and Crick (1953*b*) in which the molecule divides longitudinally, each half being conserved and acting as a template for the formation of a new half.

Site The position occupied by a mutation within the gene. (Cf. Locus.)

Strand It is customary in genetics to use strand for chromatid. It has therefore been used in that sense in this book, where it has also been used for one double-chain (that is, duplex) DNA molecule. Chain, but not strand, has been used for a single polynucleotide.

Structural change A general term for a deletion, duplication, or inversion of a chromosome segment, or an interchange of segments which may be between non-homologous chromosomes.

Synapton The term used in this book as a shortened version of synaptinemal complex. This expression was introduced by Moses (1958) for the structure which he discovered in electron micrographs of paired chromosomes at the pachytene stage of meiosis. It is derived from the word synapsis (Gk. union) which has been used by some authors for chromosome pairing.

Telophase The last stage (Gk. *telos*, end) of mitosis, or of either division of meiosis, during which the chromosomes become progressively thinner and more elongated (Heidenhain, 1894). Telophase is said to begin with the formation of a nuclear membrane round each group of daughter-chromosomes.

Test-cross A cross between a heterozygote and the corresponding homozygous recessive, for example, $\frac{A\ b\ C}{a\ B\ c} \times \frac{a\ b\ c}{a\ b\ c}$, where A/a, B/b and C/c represent three pairs of alleles, and the horizontal lines separate the contributions from the two parents of each individual.

Tetrad The four haploid cells formed at the end of meiosis. The term was

formerly used for the four chromatids making up a chromosome-pair at the first division of meiosis.

Trans The term proposed by Haldane (1941), by analogy with chemical isomerism, to describe a double heterozygote in repulsion phase, that is, with the dominant factors derived from opposite parents. The term is used chiefly for closely-linked mutants, to which it was first applied by Pontecorvo (1950). The trans configuration for two recessive mutants, 1 and 2, is $\frac{+2}{1+}$, where + stands for the wild-type, and the line separates the contributions from the two parents. (Cf. Cis.)

Transcription The transfer of information in the form of nucleotide sequence from DNA to RNA.

Transduction Gene transfer from one cell to another brought about by a virus. The phenomenon was first described by Lederberg, Lederberg, Zinder and Lively (1952) with reference to *Salmonella typhimurium*, fragments of the hereditary material of which may be transferred by virus *P*22.

Transformation Gene transfer from one cell to another brought about by fragments of the hereditary material of a cell persisting after its breakdown and subsequently entering another cell. The phenomenon was first described by Griffith (1928) in *Diplococcus pneumoniae*.

Transition The term proposed by Freese (1959) for a mutation caused by the substitution in DNA or RNA of one purine by the other, and similarly with the pyrimidines. (Cf. Transversion.)

Translation The transfer of information in the form of nucleotide sequence in DNA or RNA into amino-acid sequence in a polypeptide.

Translocation Transfer of a segment of a chromosome to a non-homologous chromosome. Translocations are usually reciprocal. (Cf. Interchange.)

Transversion The term proposed by Freese (1959) for a mutation caused by the substitution of a purine for a pyrimidine, and *vice versa*, in DNA or RNA. (Cf. Transition.)

Trisomic The term proposed by Blakeslee (1922) to describe an otherwise diploid individual which has one of the chromosomes represented three times instead of twice.

Wild-type The normal condition, either with regard to a whole organism (wild-type strain), or with reference to a particular mutation (wild-type at that locus or site, denoted by a plus sign).

Zygotene The term proposed by Grégoire (1907) to describe the nuclei at a particular stage of prophase I of meiosis when the homologous chromosomes are associating side by side. (Gk. *zygon*, yoke; *taenia*, ribbon.)

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