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GENETICS
and
METABOLISM

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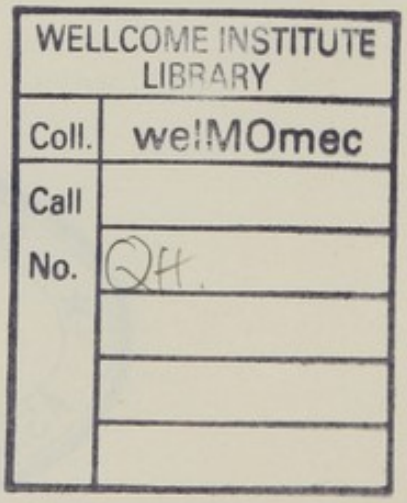

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· · · inheritance is the recurrence,
in successive generations, of like
forms of metabolism.

E. B. Wilson, 1896

Preface

The purpose of this book is to bring together a variety of facts and ideas derived from the fields of genetics and biochemistry, and, to a lesser extent, from those of physiology, cytology, and embryology, in an attempt to synthesize a general picture of the biochemical basis of inheritance. It is our thesis that inheritance is characterizable in terms of transmission of control of relative rates of biochemical reactions within complex and interlocked metabolic patterns. This is not a new idea, since it has been anticipated in the thinking and by the experimental results of a number of investigators. But even now, in spite of the compilation of a great deal of pertinent experimental data, the ambitious objective of providing complete factual support for the above thesis has not been achieved in this book, as should be readily appreciated from the fact that the title is "Genetics *and* Metabolism" rather than a more pretentious one which would at least indicate that the subject matter discussed is well enough integrated to be described by a single word. In our opinion, such a complete integration has not been attained, nor is it attainable at the present time with present data. It can be shown that a variety of fascinating apparent relationships exist, but more facts are needed to demonstrate either that they are significant or that we have been misled by false clues. Nevertheless, we believe that progress has been such that the time is ripe to present, within the confines of a single volume, a collection of data that have a direct bearing on the problem.

This book has been written primarily for advanced undergraduate or beginning graduate students of genetics, biochemistry, or microbiology. A background of elementary biology, genetics, and biochemistry is assumed. However, in order to make the contents of the book more accessible to more individuals, a short discussion of elementary genetics is given in the first chapter, and some elementary facts of biochemistry and general biology are included where they seem most pertinent.

Although many of the facts discussed are those which may also receive attention in courses in genetics and biochemistry, they are

not, in general, those that occupy central positions of emphasis in formal courses in either subject. Nor are they usually presented in juxtaposition so that the relationships between them become obvious. It is for these reasons that we feel that the material contained herein is not repetitious of that covered in other textbooks.

Material encompassing the general principles included here, with supplements from current advances in the field, have been presented to senior and graduate students in biology and biochemistry in a course given by one of us at the University of Texas for the past seven years. The response of the students has encouraged us to feel that this material provides a creative supplement to the training of those who intend to make the scientific study of life their life's work.

Robert P. Wagner
Herschel K. Mitchell

April, 1955

Acknowledgments

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The art work for the preparation of a number of the figures was done by Mrs. Grace Hewitt Groce. To her and to Miss Nina Stehr, who assisted in the preparation of the manuscript, we offer our thanks for their excellent work.

Contents

CHAPTER 1

Introduction to the Gene Concept

The discussion in this book is almost totally concerned with how genes act, or the effects of their actions, rather than with how they are inherited. But it is nonetheless necessary to know at the outset something about the meaning of the term gene, what exactly it refers to, and how the existence of genes is inferred from experimental evidence. This requires some understanding of the mechanism of Mendelism, and related phenomena associated with sexual reproduction, for it is from these that the modern concept of the gene is derived.

The gene concept is perhaps best introduced by defining the more important terms which are used to describe it. The original definition of the word gene, as given in 1911 by Johannsen (317), the man who coined it, was brief and simple:

> The gene is nothing but a very applicable little word, easily combined with others, and hence it may be useful as an expression for the "unit factors," "elements" or "allelomorphs" in the gametes, demonstrated by modern Mendelian researches.

Johannsen's word filled a definite need from the very beginning of genetics, because it gave geneticists the elements of a vocabulary by means of which they could conveniently separate the idea of cause, i.e., the inherited determination and capacity to develop certain characteristics, from the effects, the characteristics themselves. From gene the term *genotype* was derived to describe the total complement of genes in the fertilized egg, and hence the total capacity to develop certain characteristics (317). To describe the end result, the appearance of the organism resulting from gene action, the term *phenotype* was introduced.

At the time that Johannsen coined the term gene there was little else certain in genetics beyond the occurrence of Mendelian ratios which were obtained when certain types of individuals were crossed. A gene

was recognized then, as it is now, only when it existed in two forms or alleles (contraction of the obsolescent term allelomorph) recognized as being different by their differential effects on the phenotype. Its possible existence as a physical entity was appreciated by only a few of the geneticists of the time, and Johannsen himself was not among them. Proof of the physical existence of genes came when it was conclusively established by Morgan, Sturtevant, Muller, and Bridges (440) that Mendel's units, called genes by Johannsen, were closely connected with visible nuclear structures known to the nineteenth-century cytologists as chromosomes. It then became possible to think of genes as inherited physical entities which express themselves by controlling or determining the processes of development which culminate in the phenotype. These workers demonstrated not only that genes are located on chromosomes but also that they have a linear arrangement on the chromosomes. With the discovery of the significance of the giant salivary gland chromosomes of *Drosophila* by Painter (468), it became possible to identify regions of chromosomes with specific genes and to prove conclusively that each gene occupies a specific *locus* on its chromosome.

1. The Mechanism of Mitosis

One of the chief requirements for an understanding of genetics is an appreciation of the significance of the basic means of cell reproduction among cellular organisms. These processes, mitosis and meiosis, are discussed below in what is thought to be enough detail to provide the uninitiated reader with a background sufficient to understand the fundamentals of the gene concept.

Mitosis is a type of cell division in which a cell gives rise to two daughter cells that possess the same number and same kind of chromosomes found in the mother cell. The process is diagrammed in Fig. 1. The essential factors in the process are: (1) each chromosome within the nucleus about to divide is duplicated—as far as can be determined by genetic means—exactly; (2) the duplicates separate and go to opposite poles of the dividing cell; (3) the distribution of chromosomal material between the two new daughter cells is generally considered to be exactly equivalent, because of the evident elegant mechanism operating to bring about an equal distribution. This is by no means true for the cytoplasm, which has a more homogeneous appearance than the nucleus and appears not to have any mechanism insuring its equal distribution. This point, concerning the distribution of the cytoplasmic

part of the cell, is not of particular importance in the present discussion, but will receive more attention when its detailed consideration becomes important in Chapter 12 in connection with the discussion of cytoplasmic inheritance.

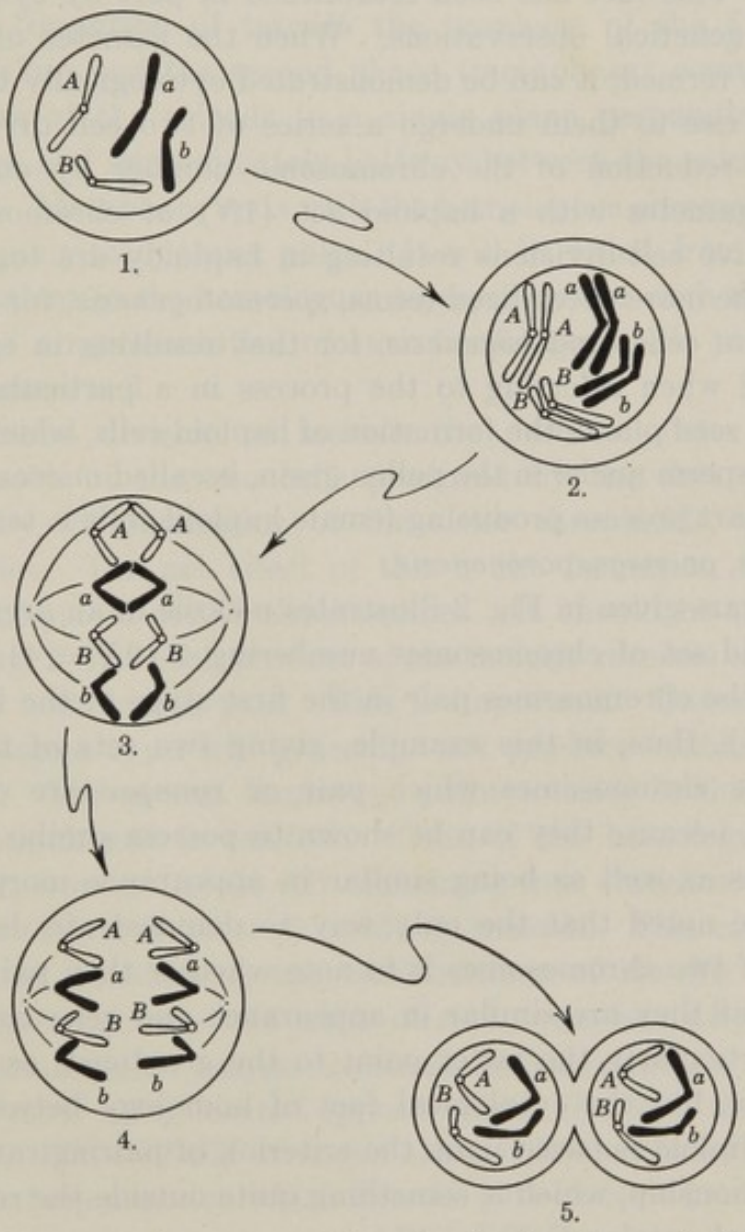


Fig. 1. A diagrammatic representation of mitosis. Some significant stages of mitosis of a cell containing four chromosomes. Stage 1. The chromosomes are visible in the nucleus: early prophase. Stage 2. The chromosomes have been duplicated: late prophase, early metaphase. Stage 3. The duplicates separate: early anaphase. Stage 4. Late anaphase. Stage 5. Telophase and end of mitosis; cytoplasm dividing.

2. The Mechanism of Meiosis

The higher organisms are for the most part diploid ($2N$), which means that they possess two similar sets of chromosomes in each of their cells. This fact has been established in part by cytological and in part by genetical observations. When the gametes of diploid organisms are formed, it can be demonstrated cytologically that the cells which give rise to them undergo a series of two cell divisions which result in a reduction of the chromosome number by one-half, thus producing gametes with a haploid set ($1N$) of chromosomes. The two successive cell divisions resulting in haploidy are together called meiosis. The more specialized terms, *spermatogenesis,* for meiosis producing sperm cells, and *oögenesis,* for that resulting in egg cells, are widely used when referring to the process in a particular sex of an animal. In seed plants the formation of haploid cells, which eventually give rise to sperm nuclei in the pollen grain, is called *microsporogenesis;* its counterpart process producing female haploid cells is termed *macrosporogenesis,* or *megasporogenesis.*

The diagram given in Fig. 2 illustrates meiosis in an organism which has a diploid set of chromosomes numbering 4 ($2N = 4$). It will be noted that the chromosomes pair in the first stage of the first division (prophase I), thus, in this example, giving two sets of two chromosomes. The chromosomes which pair or *synapse* are described as homologues, because they can be shown to possess similar or identical sets of genes as well as being similar in appearance morphologically. It should be noted that the only way to demonstrate decisively the homology of two chromosomes is to note whether they pair in meiosis. The fact that they are similar in appearance and gene content is important, particularly the latter point to the geneticist, as will be discussed below, but the cytological fact of homology between chromosomes in a diploid is based upon the criterion of pairing rather than on genetic relationship, which is something quite outside the realm of pure morphological cytology.

At the time of pairing or shortly thereafter the chromosomes can be seen to have doubled in number by each member of a pair becoming duplicated. The result is the formation of a pair of closely associated duplicates from each chromosome which are distinguished from the mother chromosome by being called sister *chromatids.* Thus each original pair of homologues becomes a *tetrad,* or a packet of four *chromatids* consisting of two sets of two identical (sister) chromatids.

The members of each pair of sister chromatids remain attached to one another during this phase of meiosis because they have only one centromere (or spindle fiber attachment) between them, for when the sister chromatids are formed, the duplication process does not extend to the centromere, at least not to an extent that becomes evident.

With the formation of tetrads the prophase of the first division draws to a close and the second phase (metaphase) commences with the lining up of the tetrads in a single plane perpendicular to the spindle fibers, and approximately halfway between the poles of the cell. (See Fig. 2.) Metaphase ends with the homologous chromosomes separating and going to opposite poles. It will be noted from Fig. 2 that this is equivalent to the homologues *segregating,* for each daughter cell then receives a representative of each homologous pair present in the original $2N$ germ cell.

In the second division of meiosis the pairs of sister chromatids present in each of the two cells produced by the first division are broken up by the separation of the sister chromatids, which go to opposite poles. The net effect of this is the formation of four cells each with a haploid set of chromosomes. It should be noted by referring to Fig. 2 that each product of the meiosis receives a representative of each homologous pair. This is important. Gametes deficient in a chromosome type will generally not produce viable zygotes on fertilization. The second important point to note is that the segregation of homologues is *random.* Thus, if the homologues differ in gene content, from a diploid cell containing four chromosomes at least four different kinds of gametes result by meiosis. This can be readily appreciated by considering the combinations of *Aa Bb* by two's in which each pair contains one letter of each type, i.e., *AB*, *Ab*, *aB*, *ab*.

When chromosomes synapse in meiosis it can be shown that they do so with like parts being located opposite like. Hence, if chromosome *A* having genes *ABCDEFGH* arranged along it in that order were to synapse with its homologue *a*, which has the gene order *a b c d e f g h*, they would do so in the following way: $\frac{ABCDEFGH}{abcdefgh}$. Like genes, or allelic genes, as will be made clear below, are, in other words, located opposite one another. Failure of the apposition of like parts to take place results in absence of synapsis and the breakdown of the meiotic mechanism.

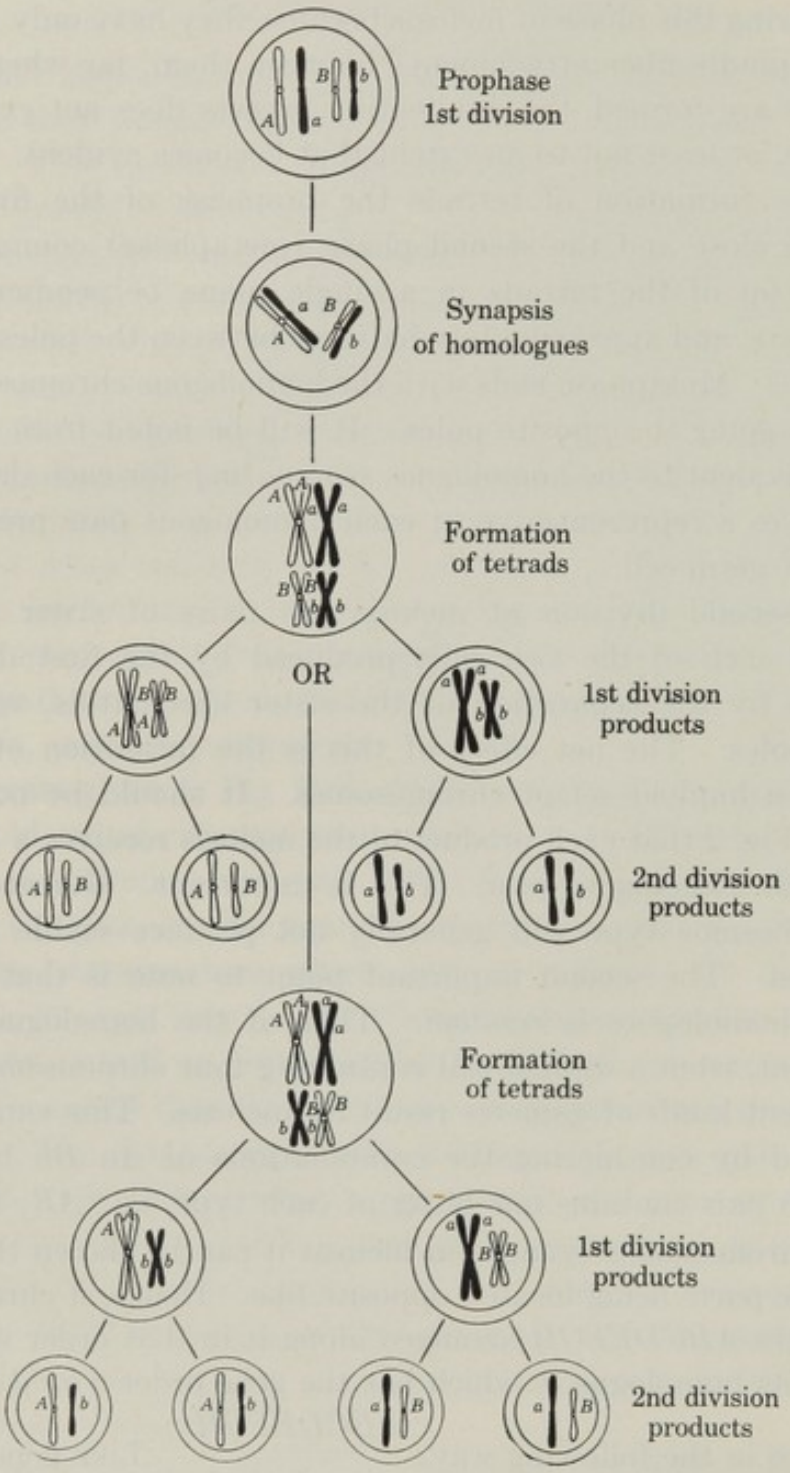


Fig. 2. A diagrammatic representation of meiosis. Meiosis is an extremely complicated process, and this figure attempts only to indicate how the products are derived. The process of crossing over illustrated in Fig. 4 and discussed on p. 11 is part of the process of meiosis, although not so indicated in this diagram.

3. Mendelism

The phenomenon of Mendelism is recognized by the occurrence of certain ratios of phenotypes of offspring. It is the direct result of meiosis with its random segregation in the parents, as described above, and of the random reconstitution of diploid offspring by fertilization, as described below.

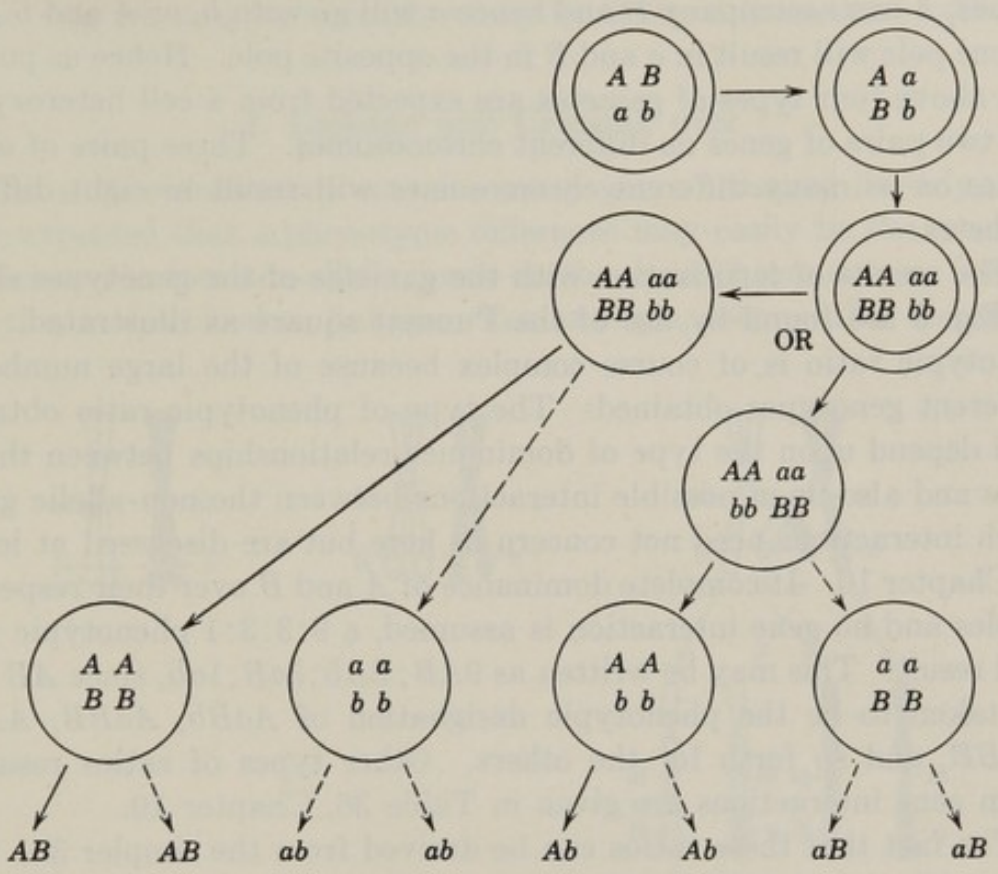
When an individual breeds true for a certain characteristic (i.e., inbreeding to its siblings or family stocks produces more of the same phenotype in the offspring without exception), it is *homozygous* for that characteristic. The term *homozygous* refers to the genotype. It means that an organism so described not only possesses the genes for the characteristic but also possesses identical kinds of genes on both sets of homologues. A homozygous individual produces, as the result of meiosis, gametes which are identical with respect to the genes for which it is homozygous. A homozygote when bred to another with the same genotype will produce offspring identical to the parents and one another. This statement may be written in genetic shorthand: $AA \times AA$ (where A denotes a gene giving a particular characteristic) gives in the F_1 (first filial generation) AA offspring.

Two individuals with different phenotypes when bred together very often give offspring identical to one of the parents. If both parents are homozygous for their respective genes controlling these characteristics, the cross may be written as $AA \times aa$. By this means it is stated that one of the parents, AA, is homozygous for A, and at the time of meiosis one pair of its homologues could be marked thus, $\frac{A}{A}$. The genotype of the second parent may be written $\frac{a}{a}$. Now, if these genes are assumed to occupy equivalent positions on homologous chromosomes and hence are alleles, it is evident that the gametes produced by parent AA will be A, those by parent aa will be a, and the F_1 offspring will be $\frac{A}{a}$ or simply Aa. These offspring are described as *heterozygous* for the genes A and a, which is another way of saying that they are not pure breeding, since they will produce two types of gametes, A and a, in equal numbers as a result of the segregation of the homologues in meiosis. If the F_1 offspring are inbred, therefore, three kinds of genotypes will appear in the F_2 generation, AA, Aa, and aa. As a

result of random fertilization of equal numbers of *A* and *a* eggs by equal numbers of *A* and *a* sperms, these three genotypes should occur in the ratio of $1AA:2Aa:1aa$.

It was stated above that in this example the F_1 offspring are identical to one of the parents. Assuming that *Aa* heterozygous individuals are phenotypically identical to the parents designated genotypically as *AA*, then it is evident that the gene *A* masks the effect of its allele *a*. The more usual way to describe this effect is to call *A dominant to a*, or, to put it another way, call *a recessive to A*. The F_2 genotypic ratio of $1AA:2Aa:1aa$ can be expressed phenotypically as a 3:1 ratio, since *AA* and *Aa* individuals are phenotypically indistinguishable. The 3:1 ratio is a *Mendelian ratio* generally described as the ratio expected from a cross between two individuals heterozygous for a pair of allelic genes both of which have something to do with the determination of the alternative phenotypes. In short, it is a ratio which when obtained tells the breeder that two alternative phenotypic characteristics are produced by allelic genes. If one allele is not completely dominant over the other, incomplete or no dominance will result, and the heterozygote will be phenotypically distinguishable from the homozygotes. This condition will be manifested by a 1:2:1 phenotypic ratio identical to the expected genotypic ratio.

The 3:1 or 1:2:1 ratios are the basic Mendelian ratios. All other ratios are derived from them. Consider, for example, two animals of opposite sex which are heterozygous for two pairs of allelic genes, each pair located on a different chromosome, with the genotype *Aa Bb*. Figure 3 illustrates the types of gametes that would be expected from meiosis in each sex with details of how these gametes are derived. It will be noted that for each female diploid cell that undergoes meiosis only one of the four haploid products survives as a functional gamete. Oögenesis in animals is identical in principle to spermatogenesis with respect to the nuclear contents, but there is an unequal distribution of cytoplasm such that one nucleus retains to the end of the second division all or nearly all of the cytoplasm. This is the functional gamete; the others are polar bodies and disintegrate in time. Since, however, it is a random matter of chance which of the four nuclei resulting from the original diploid cell receives the cytoplasm, the same types of gametes should be expected in oögenesis as in spermatogenesis provided that the genotypes of the diploid germ cells are the same. The essential factor to be recognized in connection with meiosis involving more than a single pair of homologues is that it is a matter of chance which non-homologues accompany each other in the first di-



Female gametes

Male gametes	AB	ab	Ab	aB
AB	AABB	AaBb	AABb	AaBB
ab	AaBb	aabb	Aabb	aaBb
Ab	AABb	Aabb	AAbb	AaBb
aB	AaBB	aaBb	AaBb	aaBB

Fig. 3. The results of the segregation of two pairs of homologous chromosomes. The chromosomes illustrated in Fig. 2 are represented here by their letters. The dotted lines indicate the formation of polar bodies which occurs during oögenesis. As indicated in the text, the principles involved in the formation of male and female gametes are identical. The Punnett square at the bottom of the figure shows the different possible genotypes obtained by crossing two individuals who are heterozygous for genes on two different chromosomes.

vision of meiosis. Thus in Fig. 3, in a cell with *AaBb* marked chromosomes, *A* can accompany *B* and hence *a* will go with *b*, or *A* and *b* going to one pole will result in *a* and *B* in the opposite pole. Hence as pointed out above four types of gametes are expected from a cell heterozygous for two pairs of genes on different chromosomes. Three pairs of allelic genes on as many different chromosomes will result in eight different gametes.

The results of fertilization with the gametes of the genotypes shown in Fig. 3 are found by use of the Punnett square as illustrated. The genotypic ratio is of course complex because of the large number of different genotypes obtained. The type of phenotypic ratio obtained will depend upon the type of dominance relationships between the alleles and also upon possible interactions between the non-allelic genes. Such interactions need not concern us here but are discussed at length in Chapter 10. If complete dominance of *A* and *B* over their respective alleles and no gene interaction is assumed, a 9:3:3:1 phenotypic ratio will result. This may be written as $9AB$; $3Ab$:$3aB$:$1ab$, since *AB* may be taken to be the phenotypic designation of *AaBb*, *AaBB*, *AABb*, *AABB*, and so forth for the others. Other types of ratios resulting from gene interactions are given in Table 36, Chapter 10.

The fact that these ratios can be derived from the simpler 3:1 ratio obtained when but a single pair of allelic genes are segregating can be appreciated by the application of some simple probability. For example, in a cross of $Aa \times Aa$, the chances of an offspring having the gene *A* (that is being either *Aa* or *AA*) are 3 out of 4, or 3/4. There is a like probability that the offspring will have *B* from a cross $Bb \times Bb$. Since *B* and *b* segregate independently from *A* and *a*, the probability that any individual will have both *A* and *B* as a result of a cross $AaBb \times AaBb$ will be $\frac{3}{4} \times \frac{3}{4} = \frac{9}{16}$. Similar rearranging gives the other components of the $\frac{9}{16}$:$\frac{3}{16}$:$\frac{3}{16}$:$\frac{1}{16}$ ratio by starting with the 3:1 ratio.

It should be clear from the preceding discussion of Mendelism that if two organisms from pure breeding homozygous stocks, but of different phenotype, are crossed and the F_1 inbred, the phenotypic results in the F_2 will determine the genotypic nature of the phenotypic differences. A definite 3:1 or 1:2:1 ratio obtained in the F_2 indicates that the difference is *monogenic,* i.e., the result of the difference in expression between two allelic genes. If on the other hand a different ratio such as 9:3:3:1, etc., is obtained the conclusion must be that more than a single pair of allelic genes is involved. Hence it can be seen that the definition of the gene rests upon the kind of Mendelian ratio obtained,

and that furthermore the number of gene pairs involved, if more than one, in any phenotypic difference may be deduced from these ratios.

4. Linkage and Crossing over

Since each chromosome contains many genes arranged linearly, it is to be expected that a phenotypic difference may easily be the result of a difference in two non-allelic genes on the same chromosome. Such a situation is described as linkage. For example, assume that the genes

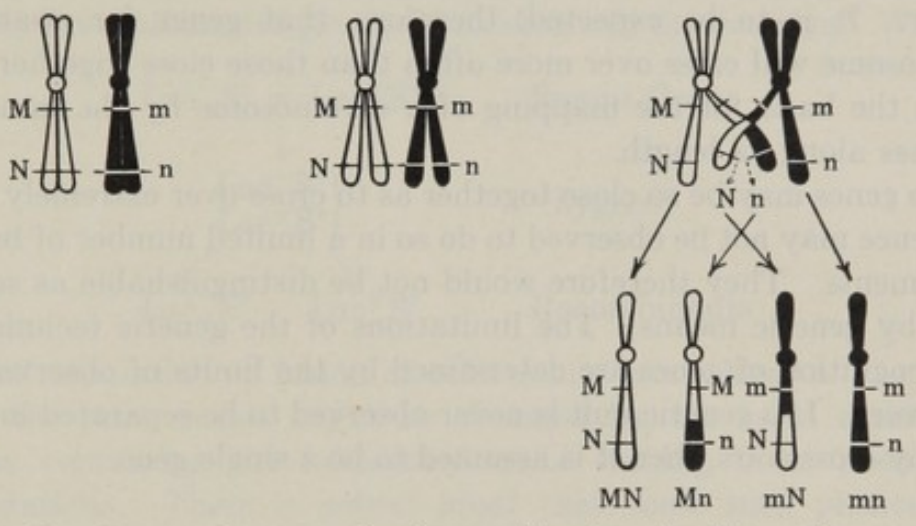


Fig. 4. Crossing over between the chromatids of a pair of homologous chromosomes.

M and N are located on the same chromosome and that m and n are their respective alleles. If two individuals, $MMNN$ and $mmnn$, are crossed, the F_1 genotype will be $MmNn$, or $\frac{M \quad N}{m \quad n}$, and, according to the information given in the preceding sections, the F_2 will consist of three types of progeny, $MMNN$, $MmNn$, and $mmnn$, in a 1:2:1 ratio. Since this is the ratio to be expected from the F_2 of a cross involving parents differing only in a pair of alleles, the conclusion must be that M and N are not recognizable as different genes. However, the integrity of the chromosomes, although maintained in substance from generation to generation, is not absolute. After the formation of chromatids in the first prophase of meiosis, and prior to the separation of the elements of the tetrad, the phenomenon of *crossing over* occurs. Figure 4 gives a diagrammatic representation of crossing over between two non-sister chromatids of a tetrad in the region between the loci of two genes M and N. The net effect of the crossover in this region is

the production of two additional types of gametes, *Mn* and *mN*, at the completion of meiosis. The formation of these types proves, furthermore, that *M* and *N* and their respective alleles occupy different loci and therefore are non-allelic genes.

Crossing over is not a rare phenomenon but a general one, and to be expected whenever homologous chromosomes pair in meiosis and form chromatids. The number of possible crossovers that can occur between the chromatids is limited by interference which certainly results in part from mechanical problems, but the points at which crossovers occur, and hence the gene loci they separate, are for the most part a random matter. It is to be expected, therefore, that genes far apart on a chromosome will cross over more often than those close together. This fact is the basis for the mapping of a chromosome by the location of its genes along its length.

Two genes may be so close together as to cross over extremely rarely, and hence may not be observed to do so in a limited number of breeding experiments. They therefore would not be distinguishable as separate genes by genetic means. The limitations of the genetic technique for the recognition of genes are determined by the limits of observation of crossovers. If a genetic unit is never observed to be separated into subunits by crossovers, then it is assumed to be a single gene.

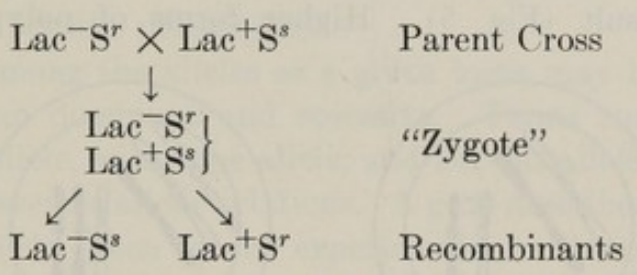
5. Recombination in General

In the previous sections considerable attention has been given to the matter of assortment of non-allelic genes in order to make clear the concept of the gene as a unit of heredity. It should also be recognized that the assortment of genes during meiosis results in recombinations. These are new combinations of non-allelic genes, which are important sources of variability as well as indicators of sexual reproduction. A strain homozygous, for example, for genes *C* and *D* remains so, unless mutation occurs (see Chapter 3), or it is crossed with a strain possessing alleles *c* and *d*. The heterozygote *CcDd* is expected to produce gametes with *Cd* and *cD* gene complements by recombinations occurring during meiosis. Recombinations can then be said to be indicators of sexual reproduction, for this is the only means by which homozygous strains may become heterozygous other than by mutation.

In the higher organisms it is unnecessary to detect recombinations in order to prove the occurrence of sexual reproduction, for fertilization is an observable event. In the bacteria, however, fusion of cells to form zygotes has never been observed, but it is known that recombinations

occur (356) and hence that some form of sexual reproduction takes place.

Certain strains of *Escherichia coli* will produce recombinants when strains of different genotypes are present together in the same liquid medium. For example, by mixing together an *E. coli* strain which is incapable of fermenting lactose and is resistant to streptomycin with another capable of fermenting lactose and susceptible to streptomycin, it is possible to obtain (1) a strain which is a fermenter of lactose and resistant to streptomycin, and (2) one which is a non-fermenter of lactose and susceptible to streptomycin. The results may be interpreted as a recombination produced as shown in the diagram. Whether



an actual copulation or fusion of cells occurs is not known. Approximately the same result might be obtained by the cells' exchanging particles containing the hereditary units causing these phenotypic manifestations. There is actual proof that some such phenomenon occurs in *Pneumococcus* (173) and *Hemophilus influenzae* (4), as described in Chapter 3. In *Salmonella*, genetic exchange resulting in recombinations has been proved to take place through the agency of bacteriophage particles carrying the genetic units from one cell to the other (724, 611). Finally, bacteriophage particles themselves have been proved to undergo recombination (273).

It is possible by recognition of recombinations (1) to determine whether a given phenotype is inherited through one or more genes, (2) to make a more precise definition of the boundaries of the gene than would otherwise be possible, and (3) to establish the occurrence of genes in organisms which give no visible evidence of sexual reproduction. Above all it should be recognized that the gene concept is derived from results of breeding experiments, and that the existence of a gene as a hereditary unit dissociated from other units is established by recombination data. Furthermore, it should be noted that the idea of one gene's being an allele of another is based upon the fact that the two never recombine. Since crossing over results in the exchange of equal and equivalent segments of chromatids, such an eventuality is not expected to occur. Actually, the fact that unequal

crossing over does occur in rare instances and can be detected and shown to give abnormal phenotypes is in itself the exception proving the rule. Further consideration is given to the matter of recombinations and the gene concept in Chapter 9.

6. Some Definitions and Symbols

Polyploidy

Occasionally a diploid cell doubles its chromosome number and becomes a tetraploid ($4N$) (Fig. 5). The gametes of a tetraploid will be diploid, and, if these fertilize haploid gametes, triploid ($3N$) individuals will result (Fig. 5). Higher forms of polyploidy such as

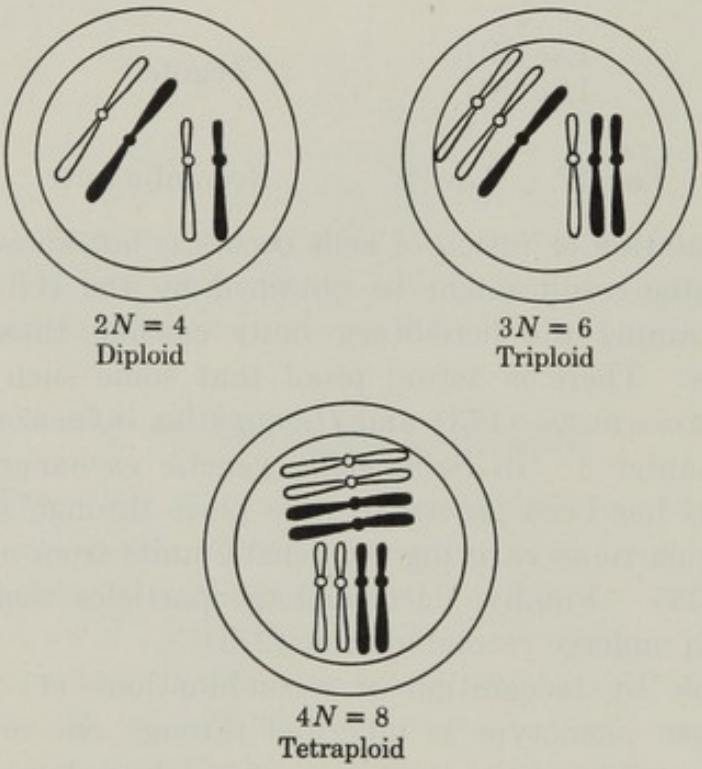


Fig. 5. Different conditions of ploidy.

hexaploidy, octoploidy, and decaploidy as well as their odd-numbered derivatives are also known to occur. As a general rule polyploidy is uncommon among animals but common in cultivated plants and in microorganisms such as yeast (520, 489) and possibly bacteria. Its significance in connection with gene action is related to the fact that in polyploid cells it is possible to have a particular gene present more than twice. This is of advantage, as pointed out in Chapters 4 and 9, when it is found desirable to study the effects of genes in different doses.

Genes and Alleles of Genes

The locus of a gene on a chromosome is its location as determined primarily by crossing-over data which can, when properly analyzed, give the approximate position of the gene relative to its neighbors on the same chromosome. At this same location, but not simultaneously, there may also exist allelic genes. Allelic genes may be thought of as different genes which occupy equivalent loci on homologous chromosomes, or, conversely, as different forms of the same gene. Both descriptions are in common usage, and both are used in the succeeding chapters. The definition of alleles as different forms of a gene is an operational one, and as such is useful, for in general alleles do have related effects upon the phenotype.

Differences among the alleles at a given locus may be described by terms other than dominant and recessive. Terms such as standard allele, mutant allele, wild-type allele, and normal allele are commonly relied upon to describe allelic relations. A gene described as "standard" is simply the allele taken by the experimenter as the one to which he compares all other alleles at the same locus. It may or may not be dominant. The "wild-type" gene is one which is found to give the "normal" phenotype in a wild or natural population. "Wild-type" and "normal" are in this sense used synonymously. Any deviation by mutation from the wild-type or normal conditions results in a "mutant" allele or gene. In general the wild-type or normal allele is dominant to the mutant.

Symbols

The symbols used by the geneticist are for the most part the letters of the alphabet. In general a capital letter designates a gene that is dominant; a lower-case letter, a recessive. If two genes are indicated by the same letter, they are alleles. Non-allelic genes which have similar or identical effects on the phenotype may be indicated by the same letter symbol, with the difference between them indicated by different subscripts. Thus w_1 and w_2 are non-allelic genes in maize (corn) both of which cause loss of chlorophyll. Their alleles (full chlorophyll) are W_1 and W_2 respectively. Capitals, lower-case letters, and subscripts are commonly used in this way by plant geneticists, as well as by mammalian geneticists studying inheritance in humans and other mammals.

A somewhat different symbolism is used by geneticists working with *Drosophila* and microorganisms such as fungi and bacteria. In these

organisms the dominant, normal, or wild-type allele is given a lower-case letter symbol with a superscript, +. Thus the gene for normal eye color in *Drosophila melanogaster* is given the symbol w^+. A mutant gene allelic to w^+ is given the symbol w (white). Other alleles at this locus are indicated by superscripts such as w^{co}, w^e, etc. A mutant gene which is *dominant* to its wild-type allele is indicated by a capital letter, and the wild-type allele by the same capital, but with a + superscript. When there is no doubt about which gene locus is being discussed, the wild-type allele is indicated simply by +. Thus the designation $w/+$ is equivalent to w/w^+.

The Gene Concept

Inheritance is, for the most part, the transmission of particulate units, the genes, located on larger units, the chromosomes. The existence of a specific gene is based upon the recognition of allelic alternative forms which in general act upon the same aspect of the phenotype, but in different degree. Each gene is a physical entity which (1) is not subdivided by crossing over, (2) has a specific function in the production of the phenotype, (3) can be duplicated and passed from generation to generation, and (4) can mutate to a changed condition.

In this statement of the gene concept no role in heredity is assigned to other parts of the cell. This does not mean that other parts of the cell do not have such a role, but simply that most of what is known today about heredity is known to be associated with the transmission of the chromosomal genes. It must be recognized that particulate units may exist in the cytoplasm which have all the properties of genes except that they are not attached to chromosomes and hence do not show Mendelian segregation (see Chapter 12). There is even a certain disposition among geneticists to refer to such entities as "cytoplasmic genes." In this book, however, the term gene will refer only to chromosomal entities which have been shown to segregate according to the laws of Mendel.

7. Life Cycles

The principles stated in the previous sections apply to all organisms which have sexual reproduction. However, their general application may be somewhat confusing unless the life cycles of the different types of organisms discussed in the book are understood. Figures 6, 7, and 8 are intended to give the reader some idea of the three principal types of life cycles found among the organisms used in the following chapters as illustrative material. These are generalized diagrams and various

species and even strains often have highly specialized variations in their structures and reproductive processes.

A good many experimental examples used in this book are derived from studies using the fungus *Neurospora crassa*, and the life cycle of

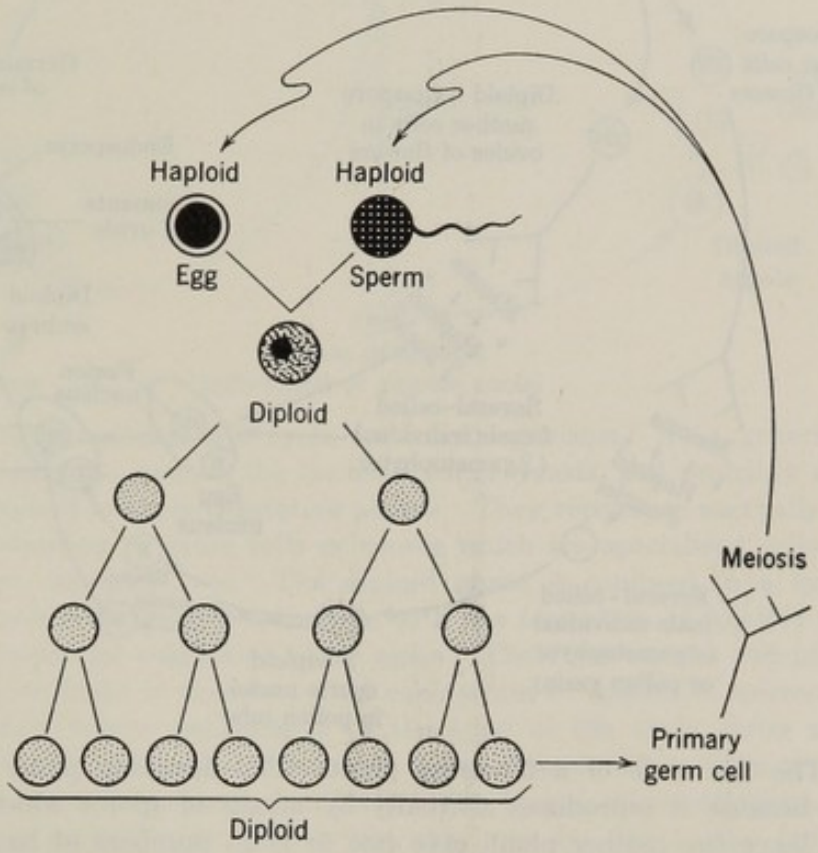


Fig. 6. A diagrammatic representation of an animal life cycle. The diploid cell (zygote) produces many cells by successive mitotic cell divisions. Most of these undergo differentiation and become body cells (soma), some of them become specialized germ cells present in the gonads. These primary germ cells are the only body cells to undergo meiosis and produce the haploid gametes.

this organism is diagrammed in Figs. 8 and 28. A characteristic of this fungus, as well as of some others, which is not shown in the diagrams, is that the vegetative mycelium contains perforated cross walls which permit a free migration of nuclei, and cytoplasmic constituents along the hyphae. Thus, discrete cells are not delineated in the mycelium, and many nuclei are present in the same cytoplasm. There is also a mechanism for hyphal fusion between different strains, and when this occurs the nuclei and presumably the cytoplasms mix, giving rise to the phenomenon of *heterocaryosis* (28). This term refers to the existence of nuclei with different constitutions in the same cytoplasm. In some respects this situation resembles that in diploidy,

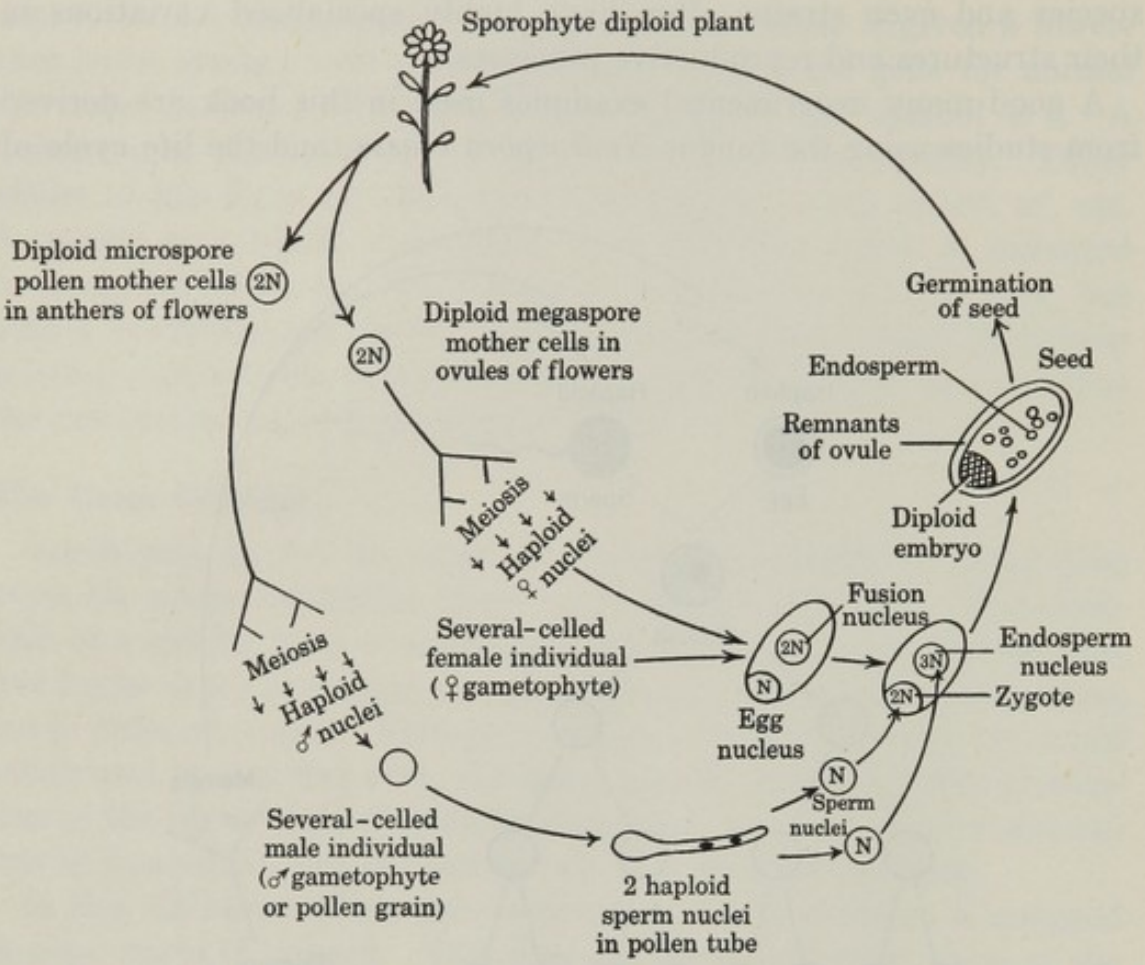


Fig. 7. The life cycle of a flowering plant. The flowering plant is called a sporophyte because it reproduces asexually by means of spores which, although they never leave the mother plant, give rise to large numbers of haploid nuclei by meiosis. The diploid microspore pollen mother cells are produced in the male parts of the flower, specifically the anthers, and give rise, as shown in the diagram, to the pollen grains. Each pollen grain upon maturing contains three haploid nuclei, two of which are involved in the fertilization process. The diploid megaspore mother cells are produced in the ovule of the ovary of the flower and give rise to specialized structures within the ovule, known as the embryo sac or the female gametophyte. The important parts of this structure are: (1) a haploid egg nucleus at one end and (2) the fusion nucleus which may be diploid or of higher ploidy toward the center of the structure. This nucleus results from fusion of two or more haploid nuclei within the embryo sac. In corn this fusion nucleus is always diploid.

Pollination consists in the pollen grain being deposited on the stigma of the female part of the flower and the growth of a tube from the pollen grain through the tissue of the stigma and the style, a structure which connects the stigma with the ovary, into the ovary where it comes into contact with the embryo sac. Thereupon two of the haploid nuclei within the pollen tube, designated as the sperm nuclei, enter the embryo sac and one of them fuses with the egg nucleus to form a zygote; the other fuses with the fusion nucleus to form the endosperm nucleus. The zygote nucleus by a series of mitoses gives rise to the embryo.

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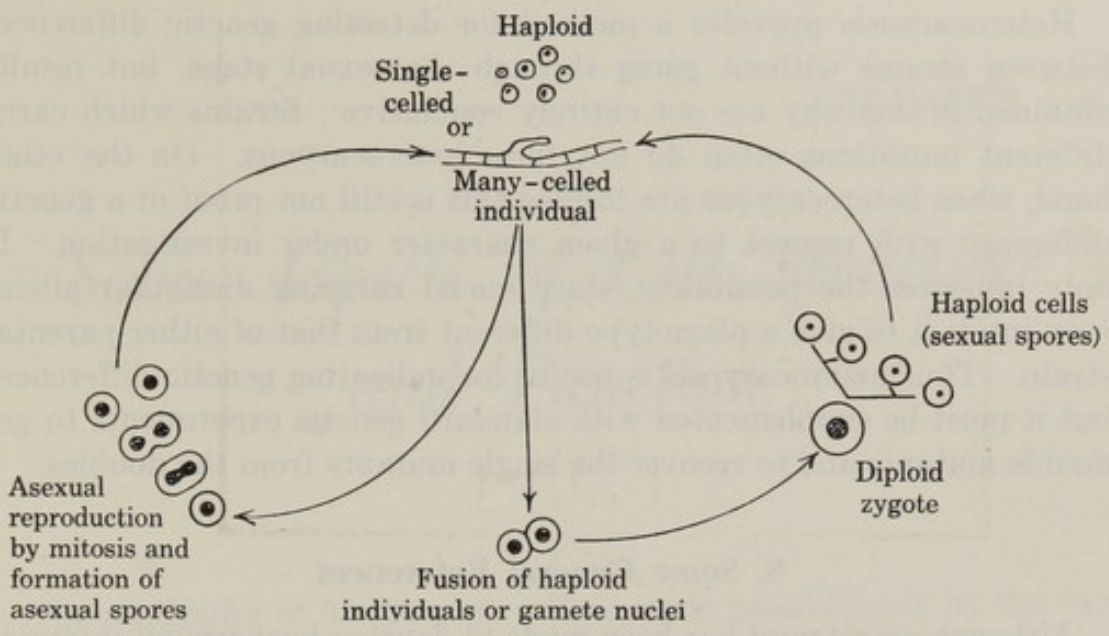


Fig. 8. A generalized life cycle for microorganisms. As a general rule, the primitive organisms such as the bacteria, fungi, yeasts, and probably most of the algae are haploid in their vegetative phases. They reproduce asexually by mitosis with the formation of more cells or spores which are specialized cells giving rise to mycelium, filaments, etc. The diploid phase is confined to a zygote which almost immediately undergoes meiosis with the formation of haploid cells, which thereupon begin the vegetative phase again. There are enough exceptions to this general rule to make it necessary that each strain or species of microorganism be closely studied before assigning it to the type of life cycle given above. For example, there is reason to believe that many yeasts and bacteria are polyploids and thus have a complicated inheritance. Many microorganisms appear to undergo only asexual reproduction but this may be due to the fact that it is difficult to study their life cycles. As stated above, certain bacteria certainly undergo the equivalent of sexual reproduction without the formation of a zygote.

with interactions between dissimilar nuclei giving results much like those in heterozygous diploids. On the other hand, in heterocaryons genetic and environmental factors have a strong influence on the ratios of nuclei that exist in the common cytoplasm, and selection in favor of one kind of nucleus or the other frequently occurs. The gene interactions in heterocaryosis necessarily take place indirectly through the cytoplasm, whereas this may or may not happen in diploidy.

The endosperm nucleus gives rise to many nuclei which together with the surrounding cytoplasm constitute the endosperm tissue. This tissue has a purely nutritive function. The embryo, the endosperm, and the remnants of the ovule within which the embryo sac develops, together constitute the seed. Upon germination of the seed, the embryo develops into a new sporophyte diploid plant. The remains of the ovary form the fruit, which is not shown in this figure.

Heterocaryosis provides a method for detecting genetic differences between strains without going through the sexual stage, but results obtained in this way are not entirely conclusive. Strains which carry different mutations often do not form heterocaryons. On the other hand, when heterocaryons are formed this is still not proof of a genetic difference with respect to a given character under investigation. It only indicates the possibility, since nuclei carrying dissimilar alleles may interact to give a phenotype different from that of either parental strain. Thus heterocaryosis is useful for indicating genetic differences but it must be supplemented with standard genetic experiments to get double mutants and to recover the single mutants from the doubles.

8. Some General References

Although an attempt has been made to develop background information throughout for the important topics discussed in this book, the reader may find it necessary to augment the explanatory material by consulting other works, either more general or more specific than this one. Among the many excellent general works on genetics which will be found particularly helpful in this connection are: those by Srb and Owen (592*a*); Sinnot, Dunn, and Dobzhansky (565); and Goldschmidt (211*a*). General and specific aspects of biochemistry are comprehensively treated by Fruton and Simmonds (189*a*). More specific information on certain modern aspects of genetics can be found in *Genetics in the Twentieth Century,* edited by Dunn (154*a*). Detailed discussions of the genetics of microorganisms are to be found in the books by Braun (70*a*) on bacteria, and by Catcheside (99*a*) on microorganisms in general. Both these books also have discussions on the biochemical aspects of the genetics of microorganisms. The field of viral genetics is thoroughly covered by Luria (384). Many provocative ideas about the relations between genetics and metabolism can be found in two stimulating works by Haldane (251, 253).

CHAPTER 2

Some Aspects of Cell Structure and Function

The continuity of life through cells is made possible only by the fact that cells have a definite organization which is duplicated at each cell division. The physical and chemical basis to this organization was understood in only the vaguest of terms until the rise of genetics in the twentieth century, when it was shown that the chromosomes maintain their integrity from cell generation to cell generation and are in large part responsible for the transmission of characteristics. Just how important the other parts of the cell are in this connection relative to the indisputably important role of the chromosomes is at present an important matter for speculation and investigation (see Chapter 11). But it is, in any case, significant that the individual cell is organized and its phenotype is a result of this organization. Any mechanistic interpretation of heredity must, therefore, necessarily be based upon the understanding of the chemical and physical nature of cell organization.

1. Common Cell Properties

All cells have certain features in common in addition to the ability to reproduce and form two daughter cells.

Communication between the cell contents and the external environment of the cell is carried on through a plasma membrane which acts both as an envelope to keep the cell contents restricted and as a selectively permeable membrane. By means of it the movement of materials into and out of the cell is controlled to a very large extent. This control extends not only to a quantitative regulation of the amount of materials moving in or out but also to a qualitative regulation as well, since the membrane may be completely impermeable

to some compounds. Because it has this capacity, the plasma membrane must be considered one of the important regulators of metabolism within the cell.

Within the membrane, cells can nearly always be seen to consist of two distinct areas, the nucleus and the cytoplasm. A further study of each of these areas reveals a high degree of organization and specialization, which has an important bearing on the problem of continuity and function (Fig. 9).

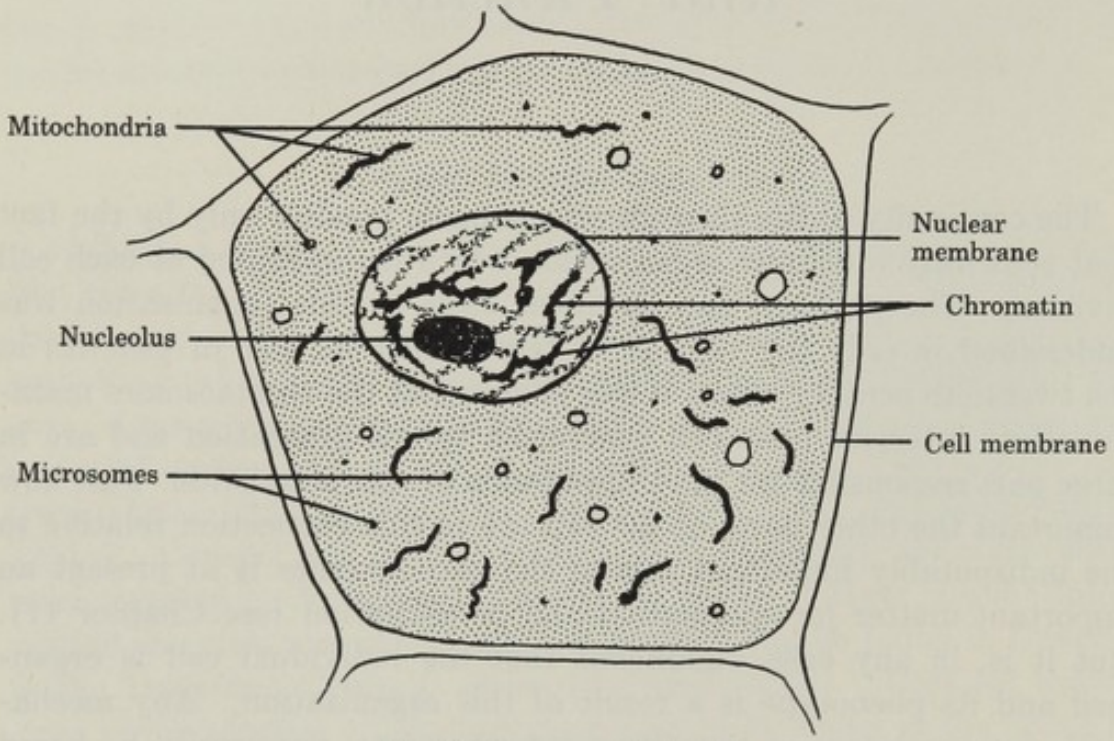


Fig. 9. A generalized cell.

Cytoplasm is that portion of protoplasm not included in the nucleus. It is separated from the nuclear contents by a nuclear membrane which has almost unknown permeability properties (7). During the process of cell division the nuclear membrane breaks down in nearly all organisms except the Protozoa. It is then reconstituted at the telophase.

The cytoplasm is ordinarily capable of existing as either a sol or a gel. In animal cells, at least the cortical part of the cytoplasm next to the plasma membrane is generally in the gel state while the inner portion has a much lower viscosity and exists in the sol form. The state of cytoplasm with respect to viscosity is a reversible one. Sol can be transformed to gel, and vice versa—an ability of the cytoplasm which enables some cells to move by ameboid movement, as well as to change and hold their shape to meet the exigencies of the environment.

Cytoplasm is a complex colloid in which the main continuous phase is water containing a considerable number of water-soluble compounds, such as carbohydrates, inorganic salts, amino acids, in true solution. Suspended in the continuous phase are a large number of different kinds of particles, ranging in size from the microscopically visible to those considerably below 0.2 μ—the approximate limit of resolution of the light microscope. This particulate phase is extremely heterogeneous and varies tremendously in composition from one type of cell to another. All cells, however, seem to possess in common two distinct types of particles, the *mitochondria* and the *microsomes*. Mitochondria range upward in size from the limits of the resolving power of the light microscope (ca. 0.2 μ) to several microns in length or diameter. Their shape varies with their source; they may be rod-shaped, in the form of filaments, or approaching the spherical. In some cells they appear to be motile, a property associated with the well-known phenomenon of protoplasmic streaming. Functionally, they appear to be centers of certain phases of oxidative metabolism (282). The structure and function of mitochondria are considered in more detail in Chapter 6.

Microsomes are submicroscopic particles ranging in size from 50 to 200 mμ (107). Some controversy exists as to whether they exist as such at all, but the evidence for a material in the cytoplasm with the physical and chemical properties ascribed to them as particles seems incontestable. They appear to have enzymatic activity, since the concentrated microsome fraction of cells obtained by centrifugation shows a high TPN and DPN dehydrogenase activity (544). As for chemical constitution, they contain a relatively high concentration of ribonucleic acid, phospholipids, and protein. According to Claude (108, 546) the greater part of the ribonucleic acid of the intact cell is bound up in the microsomes. Mitochondria and microsomes are two types of particles found in the cytoplasm as common constituents of all cells, but they are almost certainly not the only ones. Differential centrifugation of many different cell types demonstrates that there is a whole array of cytoplasmic entities ranging in an almost continuous spectrum of size and density from the subcolloidal- to the colloidal-sized particles and, finally, to microscopically visible particles such as mitochondria. The precise nature of these particles depends to a very large degree on the type of cell. As cells differentiate there appears to be a concomitant change in the particles (544), and it is to be expected, therefore, that the particulate aspects of the cytoplasm are different in the different tissues.

Animal cells in general differ somewhat from plant cells in their cytoplasmic inclusions. Golgi bodies and central bodies, the former with an unknown function and the latter with a function in cell division, are typical animal-cell cytoplasmic entities. Golgi bodies are never found in plant cells; central bodies are found only in the cells of the non-vascular plants. On the other hand, cells of photosynthetic plants invariably, except for the blue-green algae and photosynthetic bacteria, contain plastids of various types, of which the most noticeable under the microscope are the chloroplastids containing chlorophyll and other necessary parts of the photosynthetic mechanism. Other particles, many of them in the range of visibility, such as lipid droplets, glycogen, melanin, starch, and volutin (possibly metaphosphate) (379) granules, crystals of purines, uric acid, etc., may also be found suspended in the cytoplasm along with the mitochondria. Seldom, if ever, however, are they all found in the same cell, but are characteristic of the different cell types.

The continuous phase of the cytoplasm contains a large number of enzymes which may or may not be homogeneous in concentration through the cytoplasm. Presumably many of the substances found in the cytoplasm which exist as small molecules are continuous throughout the cell, since they may be expected to pass through the nuclear membrane.

Because of its content of chromosomes, the nucleus has received more attention from the cytologist and biochemist with respect to its specific physical and chemical characteristics than any other protoplasmic constituent. Some of their findings are discussed in the following section.

2. The Nucleus

The nucleus is a highly organized structure containing a number of parts that are cytologically recognizable. These include chromosomes, nucleoli (see Fig. 11), spindles, and nuclear membranes, but the relative prominence of these structures is dependent on the time of observation, since nuclei go through regular cycles during division. For this reason a generalized description of nuclei in terms of physical continuity and chemical composition is not possible. Nevertheless, there are available a good many analytical data that are descriptive of cell nuclei and their components.

This material is derived in part from studies of nuclei obtained from non-dividing or slowly dividing cells, such as chicken erythrocytes, mature sperm, or liver cells from adult animals. From these it is

practicable to isolate nuclei in quantities suitable for chemical analyses and to separate some of the nuclear components as reasonably homogeneous preparations (150). Bulk analyses of such preparations have provided very useful information, especially in combination with results obtained by optical methods and microtechniques. For example, bulk analyses show that nuclei contain quantities of desoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. Both types of nucleic acid absorb strongly in ultraviolet light, and by observations of nuclei, even in living cells, it is possible to make estimates of concentrations of these substances as well as to map their localizations within the nuclei (94).

The proteins absorb much less strongly in the ultraviolet, and their localization and concentrations can better be ascertained by staining or by staining techniques combined with enzyme or chemical treatments in fixed tissues or isolated nuclear material (323, 324, 407, 414, 558). These optical-chemical methods can also be used with nucleic acids (323, 414). Thus, bulk analysis provides information on the nature of the chemical components of nuclei and serves as a basis for predicting what kinds of stains and reactions are useful for studying the physical continuity and the localization of specific chemical components within the nuclei, as observed with the microscope in either visible or ultraviolet light.

The Composition of Whole Nuclei

Two general procedures have been utilized for the isolation of nuclei from a variety of tissues. The first makes use of organic solvents and fractional centrifugation of cellular material obtained from dried and thoroughly ground tissue (150). Benzene and chloroform and mixtures thereof are commonly used. The second method also depends on fractional centrifugation, but the nuclei are obtained from moist tissues, using an acidic to neutral citrate buffer as a suspending medium. Both methods yield reasonably homogeneous preparations of nuclei that are microscopically recognizable as such. Neither method, however, can yield nuclei of the native state, since those isolated in organic solvents lose materials soluble in these solvents and those isolated in aqueous solvents lose those substances soluble in the buffer. In addition, some protein denaturation undoubtedly takes place in both procedures. One other significant point is concerned with the possibility of adsorption of cytoplasmic components on the nuclei. This problem has given rise to considerable controversy, particularly in relation to the enzyme constitution of isolated nuclei (150).

Except for sperm cells in which there is very little cytoplasm, nuclei in general occupy about 10 to 20% of the cell volume and contain about 15 to 25% of the cell nitrogen (544). A prominent constituent of the nitrogen-containing nuclear material is desoxyribonucleic acid (DNA). This substance accounts for something in the order of 10 to 15% of the dry weight of nuclei isolated from liver in aqueous solution (414, 544), and it amounts to about 2.4×10^{-9} mg of DNA per nucleus (fowl liver cells). Under normal circumstances (exclusive of virus and phage infections) DNA is found only in cell nuclei, where it is associated with chromosome structures. In a given species each cell contains DNA in a remarkably constant proportion to the chromosome complement of the cell (49, 415). For example, liver and erythrocyte cells of domestic fowl were found to contain 2.39×10^{-9} and 2.34×10^{-9} mg of DNA per cell respectively while the haploid sperm from the same species were found to contain 1.26×10^{-9} mg per cell or just about half that of the diploid tissue. Certain oöcytes appear to provide exceptions to this rule (662). Nevertheless, there are now sufficient data to indicate that this constancy of DNA concentration is a real phenomenon. Although the DNA level is quite constant in the nuclei of one species of organism it varies over a wide range for different species (0.12×10^{-9} to 160×10^{-9} mg per cell), and there appears to be a relation between increase in cell size and increase in DNA content.

A great deal of information is now available on methods for isolation of DNA from tissues or nuclei as well as on the analysis and structure of DNA molecules (77, 105, 405, 566, 570, 678). Here it must suffice to say that DNA has a molecular weight of the order of 5 to 10 million; it is made up of probably coiled chains of nucleotides, each containing a heterocyclic base, a desoxy sugar, and a phosphate ester, linked as shown in Fig. 10. The heterocyclic base can be adenine, guanine, thymine, cytosine, 5-methylcytosine, or, rarely, 5-hydroxymethylcytosine in phage. A typical example of the proportions of bases given as moles per 100 moles of phosphorus (calf thymus DNA) is: adenine, 27.6; guanine, 23.5; thymine, 28.0; cytosine, 19.7; 5-methylcytosine, 1.2. These proportions vary appreciably in different kinds of DNA but usually not much further than this from 25 for the first four bases. It is commonly found that when adenine is relatively high thymine is also. Work done on determining the order of the purine and pyrimidine bases in the DNA chains shows an apparent randomness. That is, the bases do not exist in simple repeating units of tetra-

or pentanucleotides although it seems likely that in DNA from a given species the bases are present in a definite order.

All nuclei investigated contain ribonucleic acid (RNA) as well as DNA. The concentration of this component is variable even in nuclei from the same tissue, and only part of the total RNA of the cell is contained in the nuclei. Estimates of the proportion contained in

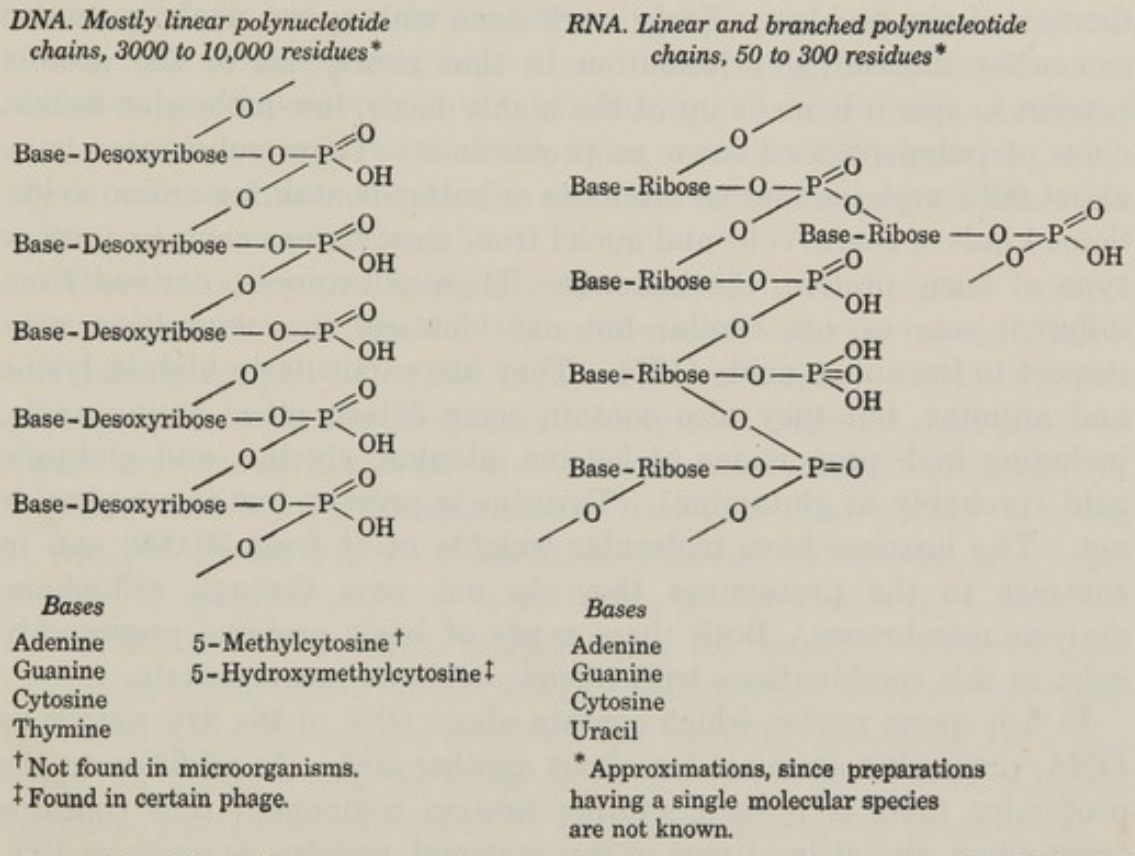


Fig. 10. Similarities and differences in desoxyribonucleic acids (DNA) and ribonucleic acids (RNA).

nuclei vary from about 3 to 35%, corresponding to a range of approximately 2 to 20% of the dry weight of nuclei. Part of this wide variation is due to differences in preparative and analytical methods, but on the other hand part of it is certainly due to real physiological differences resulting from changes during the nuclear cycle and gross metabolic differences in tissues. The ribonucleic acids are not as well known chemically as the desoxynucleic acids, and there is probably a greater diversity in molecular species and size and structure in this group. These nucleic acids contain chains and branched chains of nucleotides in which the heterocyclic bases are adenine, guanine, uracil, and cytosine. Both DNA and RNA are found combined with proteins in the native state.

Nuclei, in general, have a prominent lipid component amounting to about 3 to 10% of their dry weight. Relatively little is known about the chemistry of these substances, but it has been reported that they are predominantly phospholipids, including lecithins and cephalins (544).

The picture with respect to the protein content and composition of nuclei is a very complex one, and much remains to be done in clarification of the problem. Early work done with sperm nuclei provided somewhat misleading information in that nearly all of the protein present in sperm is made up of the highly basic, low-molecular-weight class of polypeptides known as protamines. These substances have about 90% arginine and no aromatic or sulfur-containing amino acids. Some kinds of sperm cells and nuclei from most tissues contain another type of basic protein, the histones. These substances, derived from different sources, are similar but not identical in composition with respect to the amino acids (127). They are particularly high in lysine and arginine, but they also contain some fifteen other amino acids, including high proportions of leucine, alanine, glycine, and glutamic acid (probably as glutamine). Tyrosine is present, but tryptophan is not. The histones have molecular weights of at least 30,000, and in contrast to the protamines they do not pass through cellophane dialysis membranes. Both these types of basic proteins presumably exist in salt combinations with nucleic acids as nucleoprotein.

In fish sperm nuclei, which contain about 60% of the dry weight as DNA, protamine accounts for about another 20%. In addition to the protamine there is present another protein component that contains tryptophan, and at least part of this material, perhaps as much as 10% of the nuclear weight, appears to be a fibrous protein that constitutes part of the chromosome threads. With nuclei other than those from sperm it is very difficult to evaluate the results that have been obtained regarding the amounts and kinds of proteins present. Nuclei from thymus, liver, and kidney tissues (and probably all tissues that lack protamine) contain histones in amounts perhaps of the same order as the DNA present. In addition these nuclei have present a protein containing ca. 1% tryptophan, which is present in a variable concentration and appears to be especially important in relation to chromosome structure (p. 30). This component varies in amount from about one-fifth to one and a half the quantity of DNA present.

In addition to these proteins, nuclei contain enzymes, but the picture here is complicated by problems concerned with experimental techniques. In the first place nuclei prepared by separation of dried

materials by means of organic solvents lose as much as 60% of their dry weight when they are extracted with aqueous buffers, and a good deal of this extracted material is protein, perhaps soluble enzymes or inactivated enzymes. It has been reported that liver nuclei prepared even in aqueous suspensions contain aldolase, D-amino acid oxidase, arginase, enolase, esterase, acid and alkaline phosphatase, phosphorylase, lactic acid dehydrogenase, and uricase (150). In the same preparations, catalase and succinic acid dehydrogenase activities were not found. Of those showing activities present, only phosphorylase had a higher specific activity in nuclei than in the whole cell. Activities of the other enzymes were of the same order in the nuclei as in the whole cell, and thus the nuclei actually contained only a relatively small proportion of the total.

Other investigations using nuclei prepared by the organic solvent method provide evidence for the existence of esterase, phosphatases, and nucleoside phosphorylase in a variety of tissues (609). Specific proteins such as hemoglobin, muscle myoglobin, arginase, and pancreas lipase were found in the nuclei of certain tissues only. For example, arginase, which is high in mammalian liver and avian kidney, is found in the liver nuclei but not in the kidney nuclei. Esterases, which are present in most tissues, were found in the nuclei from some tissues but not in others. The results suggest that the nuclei of differentiated tissues have also been differentiated. It seems reasonable and probable that nuclei should contain enzymes, but some investigators have cast doubt on findings such as those given above on the ground that the enzymes were merely adsorbed on the nuclei during the preparation.

The problem of composition of nuclei with respect to low-molecular-weight water-soluble substances is much like that for the soluble enzymes. Results obtained from analyses for Ca^{++} and Mg^{++} in nuclei from thymus tissue prepared by the non-aqueous solvent method gave values of 1.35% and 0.08% respectively. These values are double or more than those for the whole tissue (692). Similarly, nuclei prepared by this general procedure were shown to be particularly high in some of the vitamins following enzyme digestion of nuclei from beef heart (310). It was found that these nuclei contained from three to four times as much nicotinic acid, pantothenic acid, riboflavin, thiamine, and folic acid per unit of dry weight as did the whole tissue. Pyridoxin concentration was about the same in the two preparations, whereas the whole tissue was found to contain more biotin and inositol per unit weight than the preparation of nuclei. The total amount of

vitamin was, of course, much greater in the whole tissue than in the nuclei.

There still remains much to be done concerning the chemical composition of nuclei, particularly in relation to substances having to do with metabolic activity within these structures. They do contain free amino acids to about the same extent as the cytoplasm, but in general they do not appear to have a store of polysaccharide as a source of energy nor do they carry any apparent terminal oxidase system. Nevertheless, it is highly probable that a good deal of intermediary metabolism does take place in the nucleus in connection with the various chemical changes that are known to occur.

The Composition of Nuclear Components

The nuclear structures that have received the most attention from the standpoint of chemical composition are the chromosomes. There are two good reasons for this. First, as already discussed, a combination of genetic and cytological techniques has demonstrated that genes are associated with chromosomes, and, even though this does not prove that the genes actually are made up only of the substance that can be seen, this is an adequate reason for a disproportionate interest in the chromosomes. A second factor is the purely methodological one. More work is done on chromosome chemistry because methods are available, and because more work is done more techniques become available. It is to be hoped that this pyramiding of effort is now well started with respect to nuclear components other than chromosomes, since it is likely that all these structures are essential to the active processes of heredity.

Information on chromosome chemistry is derived from observations of living cells in the ultraviolet microscope, from cytochemical procedures on fixed tissues or isolated nuclei, and from bulk analysis of isolated chromosomes. There is general agreement that chromosomes contain DNA, RNA, a basic protein (histone or protamine), and fibrous tryptophan-containing protein. They may also contain other substances in small amounts. There is evidence, for example, that chromosomes are enclosed in a thin membrane, and this may be at least partly lipid in nature. In addition, inorganic ions, low-molecular-weight organic compounds, and even enzymes may well be present in significant quantities. It is important to note that a biologically significant quantity of a substance may be a very small amount indeed, perhaps only a few thousand molecules or less, in relation to some processes of gene functions such as in the production of two genes from one.

Structurally, the chromosomes consist of spiraled threads with characteristic beadlike bodies (chromomeres) in definite positions along the threads. These contain high concentrations of DNA and basic protein. The continuity of the thread itself appears to depend on the presence of the tryptophan-containing residual protein, although ap-

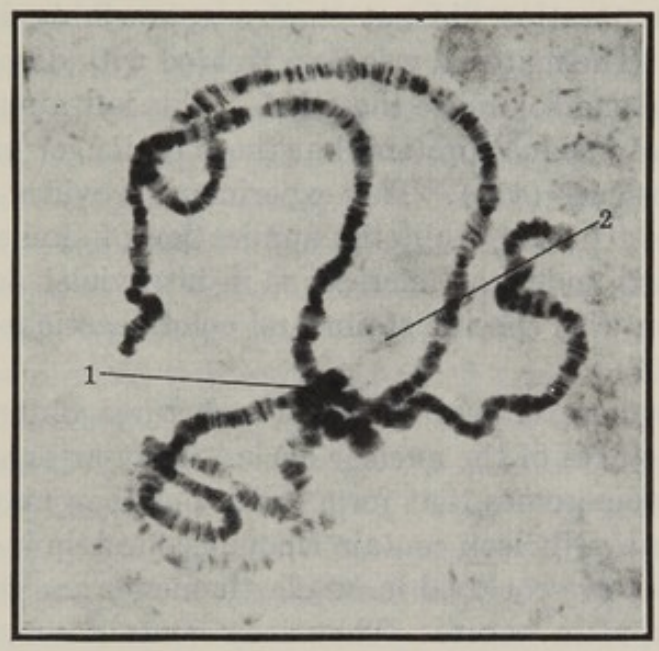


Fig. 11. The salivary gland chromosomes of a salivary gland cell of *Drosophila virilis*. These chromosomes, found only in the Diptera (flies), show details not found in any other chromosomes because of their giant size. Notice the banding. The dark bands indicate regions of high concentration of desoxyribonucleic acid. The chromosomes are attached together in the region of their centromeres in a structure known as the chromocenter (1 in the figure). The nucleolus is also in this general region (2 in the figure). The main heterochromatic parts of these chromosomes are in the region of their centromeres. The other areas, where the banding is most evident, are, for the most part, euchromatin. (This photograph was made by Dr. Frances Clayton, University of Texas.)

preciable amounts of DNA in combination with basic protein may be distributed along the entire thread. The positions of ribonucleoproteins in the chromosome structures are not entirely clear, although they are reputed to be concentrated in heterochromatic regions along with excess DNA and basic protein. Heterochromatin is the region of the chromosome that remains relatively thick and thus stains heavily and is optically opaque in ultraviolet light. The remainder of the chromosome is generally referred to as the euchromatin. As a general rule each chromosome has its characteristic amounts of euchromatin and heterochromatin arranged in a definite order along its length (Fig. 11).

Much of the work on identification of the chemical components in specific parts of the chromosomes has been done by treatment of fixed tissues or isolated chromosomes with specific enzymes or reagents for extracting specific substances. As an example, a chromosome preparation from liver was treated at *p*H 2.8 to extract the basic histone. Very little DNA was removed, and the microscopic appearance of the chromosome preparation did not change appreciably in spite of this loss of protein. The material was then treated with desoxyribonuclease to depolymerize and solubilize the DNA. This left a mass of insoluble coiled threads of residual protein, but these no longer had the appearance of chromosomes (414). This experiment provides a gross picture of chromosome structure, while the application of similar principles to materials studied under the microscope in ultraviolet and visible light (in combination with specific stains and color reactions) has provided details (324, 407).

Nucleoli are more or less spherical structures observable in most nuclei at some stages of the nuclear cycle. They arise at definite positions on the chromosomes that form them, but they may subsequently become detached. Nucleoli contain ribonucleoprotein in a high concentration, and they are enclosed in an elastic membrane that is probably at least part lipid in nature. They may contain enzymes and other substances of consequence in metabolic processes, but little detailed information is available.

During the division of the cell, the chromosomes appear to be pulled toward opposite poles of the cell by the contraction of spindle fibers. The physical existence of these fibers is well established, and it is now known that they are protein in nature, having been isolated from dividing sea urchin eggs and characterized chemically by Mazia and Dan (408). These fibers attach at specific places on each chromosome known as the *centromeres,* which frequently can be distinguished cytologically from the rest of the chromosome. Generally each chromosome has one centromere, but cases are known in which several are present or the centromere is diffuse along the length of the chromosome. The evidence that the spindle fiber protein is rich in sulfhydryl groups (408) lends considerable credence to the theory of Rapkine (408) that the contraction of these fibers is due to the oxidation of the sulfhydryls with the formation of disulfide bonds resulting in the shortening of the protein fibers.

All particulate nuclear inclusions lie in a liquid of unknown composition, and the whole is enclosed in the nuclear membrane of protein and phospholipid. The exact nature of these components is not known,

nor is its permeability understood, as pointed out above. There is good evidence that the nuclear membrane of the amphibian ovum consists of a double layer, the inner one being continuous and protein or lipoprotein in nature while the outer layer is perforate and largely lipid (433).

Chemical Changes during the Nuclear Cycle

A description of the chemical composition of the nucleus or its organized inclusions at any one point in time is not in itself any more than the briefest introduction to an understanding of nuclear functions. A little more is to be gained by observations of changes in composition that occur during the nuclear cycle, and all of this is a prerequisite to discovering the actual chemical processes by which the nuclear components exert their control in determination.

It is of course obvious that during the processes of mitotic or meiotic cell divisions there must be changes in chemical composition of nuclei. The nuclei themselves change in size, the chromosomes lengthen and then contract again, nucleoli are formed and disrupted, and spindle fibers are formed and disappear. During and between these processes the necessary materials for the formation of new nuclei must be provided from some source, and it is perhaps unlikely that all of them are mobilized at the same rate. It may even be that the relative rates of mobilization of nuclear materials exert a controlling influence on the processes of nuclear division. These necessary materials, the residual protein, the basic proteins, RNA, and lipids, may be synthesized in the nucleus, or they may be produced all or in part outside in the surrounding cytoplasm. There is evidence from studies with microspectrophotometry that RNA is formed in the nucleolus (552). Tracer experiments that show a higher turnover in nuclear as opposed to cytoplasmic RNA phosphorus can be interpreted in a similar fashion. With respect to DNA and any of the proteins or other substances that are specific to the chromosomes it is evident that concentration changes must occur at the time of or before chromosome divisions. There is some evidence that DNA precursors are mobilized during interphase (550), but this is by no means firmly established.

A significant event that occurs in the division of most cells is the disappearance of the nuclear membrane. Whether this dissolves completely is difficult to tell, but the process may provide a means of interaction of the nuclear and cytoplasmic synthetic systems on a more intimate basis than when the membrane is discernible. The membrane is present when the nucleus is in interphase, but the chromosomes can-

not be seen at this time. It is clear that they are present, however, and evidence has been presented to show that they are merely swollen and thus diffuse at this time (516).

Much of the experimental work in this field is controversial because conclusions are often based on technological assumptions, and all results should be evaluated on the basis of a careful analysis of experimental methods. This is intended only as a word of caution to the tyro, and not as a criticism of the efforts that have been made in this technically complex problem, which is extremely difficult for even the ablest of chemists.

3. Cytoplasm and Nucleus

The ease with which the nucleus may be distinguished cytologically from the cytoplasm and the relative ease with which the two parts may be separated mechanically have made it possible to study both apart in detail and discover a number of marked differences. In summary, the two cell areas differ (1) in the kinds of their particles, (2) enzymatically, (3) with respect to the distribution of RNA and DNA, (4) in the mechanism of their division during cell division, and (5) in their directness of communication with the external cell environment.

The differences in the particulate components of the nucleus and cytoplasm are in large part responsible for the enzymatic differences. Although there seem to be many enzymes common to both areas, the respiratory enzymes, for example, required for the release of chemical energy for cell processes with the utilization of oxygen are found exclusively in the cytoplasm and associated with the mitochondria. The removal of the nucleus from a cell generally has no effect on its respiration, nor has any respiration been detected in an isolated nucleus.

There are by no means as many enzymes known for the nucleus as the cytoplasm, but this does not prove that the nucleus is enzymatically inactive compared to the cytoplasm. It must be realized that an enzyme is generally recognized only by its activity. This can be tested only by having a prior knowledge of its substrate. Lack of this leads to continued ignorance of an enzyme's presence.

The significant differences in distribution of the nucleic acids must be assumed to be of importance in the roles played by the cytoplasm and nucleus. Presumably, the ribonucleic acids are confined to the microsomes in the cytoplasm, but they may be found in the chromosomes, nucleoli, nuclear sap, or spindle fibers of the nuclei; the exact

distribution depends in large degree upon the stage of the mitotic cycle. DNA is so rarely to be found in the cytoplasm that its presence there is generally taken either as accidental, an artifact, or a result of the perverseness of some individuals. Speculation supported by some circumstantial evidence has assigned to the ribonucleic acids a role in protein synthesis (69, 585), and to the desoxyribonucleic acids the more vague role of keeper of the cell's biological specificity, i.e., the chemical substance making up the genes. It has been postulated that ribonucleic acids and desoxyribonucleic acids are synthesized one from the other (550). The evidence for this is scanty, and none of it direct. Proof of it would, however, be a significant demonstration of the exchange of presumably important materials between the nucleus and cytoplasm.

Perhaps one of the most fundamental aspects of the differences between the nucleus and cytoplasm is the obvious cyclical behavior of the nucleus during which the chromosomes are duplicated and equally divided between daughter cells, as compared to the relatively stable state of the cytoplasm, and its division presumably without an apparatus to insure exact equality of distribution of its parts. It is highly probable that the nucleus is the only part of the cell which must be equally distributed to maintain biological continuity, but it should not be concluded that the cytoplasm is unimportant because of this, or that qualitatively unequal division of its parts is always possible or the rule. (See Chapter 11.)

The nucleus and cytoplasm undoubtedly constitute two different metabolic systems, separated from one another, except for short intervals during mitoses, by a nuclear membrane. They are decidedly not independent systems, however. A living cell must have both parts to continue to live and reproduce. Although the removal of the nucleus from a cell may not cause immediate death, the cell cannot reproduce, nor can it differentiate. The fact that a certain amount of differentiation is possible in nucleated eggs without cell division shows that the presence of the nucleus is necessary for differentiation. On the other hand, nuclei completely devoid of cytoplasm are unable to produce cytoplasm *de novo,* or to divide. It is the whole cell which is the living system, for it is the smallest unit capable of reproducing life, and it is important to recognize that the expression of the inherited characteristics discussed in the following chapters is through the activities of cells, not merely through genes, which are parts of cells.

CHAPTER 3

Mutation

1. Mutation Defined

Mutation is an event occurring in cells which results in heritable change. The change may result in a new phenotype, or it may merely be manifested as some visible structural alteration in the chromosomes or other inherited particles within the cell. The term *mutation* describes any inherited change which is not due to segregation or the normal recombination of unchanged genetic materials such as occurs in sexually reproducing organisms. It should not be applied to non-inherited environmental modifications, which are discussed in Chapter 10.

The capacity to mutate is as important a part of the properties of genetic material as its stability. Mutation is assumed to occur in all organisms and to be the basic source of all heritable natural variation. Therefore, we shall consider here the process and its results in some detail, placing particular emphasis on those aspects of the subject which are generally believed to bear on the nature of genes and chromosomes. Only those mutations which show a Mendelian pattern of inheritance will be considered. The reader will find a discussion of cytoplasmic inheritance in Chapter 11.

A rigorous proof of chromosomal mutation is possible only in organisms with a sexual phase. However, inherited changes which may be chromosomal occur in asexual forms as well as in the somatic cells of multicellular organisms. Somatic mutations may sometimes be demonstrated to be chromosomal by carrying the mutation over into the germ line, but in the bacteria and fungi in which only asexual reproduction occurs it may only be inferred that the mutations are indeed of chromosomal origin from the results with organisms having sex cycles.

The fact that mutations do occur in unicellular asexual species and

the soma of higher forms is good evidence that the mutation process is one which involves a basic type of change not equivalent to segregation brought about by independent assortment and crossing over as part of the sexual process. Proof of the occurrence of mutations in sexual organisms may be had from experiments in which isogenic strains (all individuals completely homozygous for the same genes) are used. In these experiments the isogenic strain is isolated from any possible contamination and simply observed for a number of generations to detect any origin of inheritable change. East (155), who performed such an experiment with tobacco (*Nicotiana rustica*), found that after a period of time his homozygous population gradually reverted to the normal state of variability found in natural, inbreeding populations. By starting with a haploid strain of tomato which was doubled to produce the diploid, Lindstrom (381) produced a completely homozygous strain which he carried under close observation for a period of 10 years. In contrast to the results obtained by East, Lindstrom found a high degree of stability in the tomato diploid lines; nonetheless, spontaneous changes did arise which could only have been the result of changes in the genetic material of the chromosomes, since there was no variability to start with and the changes were inherited according to a Mendelian pattern.

The Detection of Mutations

A mutation is recognized by the appearance of inherited phenotypic change, or by visible changes in chromosome structure which may or may not be accompanied by measurable phenotypic effects. Mutant phenotypes are generally classified as either morphological or physiological. Morphological mutants, frequently called *visibles*, are of course to be considered basically physiological in origin. They are the most easily recognized mutations and therefore the most commonly used as genetic tools. Purely physiological changes in phenotype may manifest themselves as lethals—a cessation of development and growth and subsequent early death. They may also become apparent as changes in the chemical composition of the tissues or of the excretory materials, or as alterations in nutritional requirements, and have no marked effect on the life span.

In addition to mutations recognized by phenotypic change, a large number of inherited changes must be assumed to occur which are not recognized either because their expression is slight and overlooked, owing to the adaptability of the organism, or because methods of detecting them are not available. Subtle chemical modifications might

be included in this category. Furthermore, mutations occur which are undetectable soon after their occurrence. As will be discussed in Chapter 10, it is quite possible for a mutated gene to have no apparent effect on the phenotype until it is moved into a new genome, and thus a generation or more may be required before the mutation becomes evident. Obviously, then, it is quite impracticable to observe every mutational event even when the individual carrying mutations is subjected to close scrutiny.

Types of Mutations

Two general classes of mutations are recognized by geneticists. One class is associated with visible chromosomal changes, and the other with phenotypic change unaccompanied by visible change in the nu-

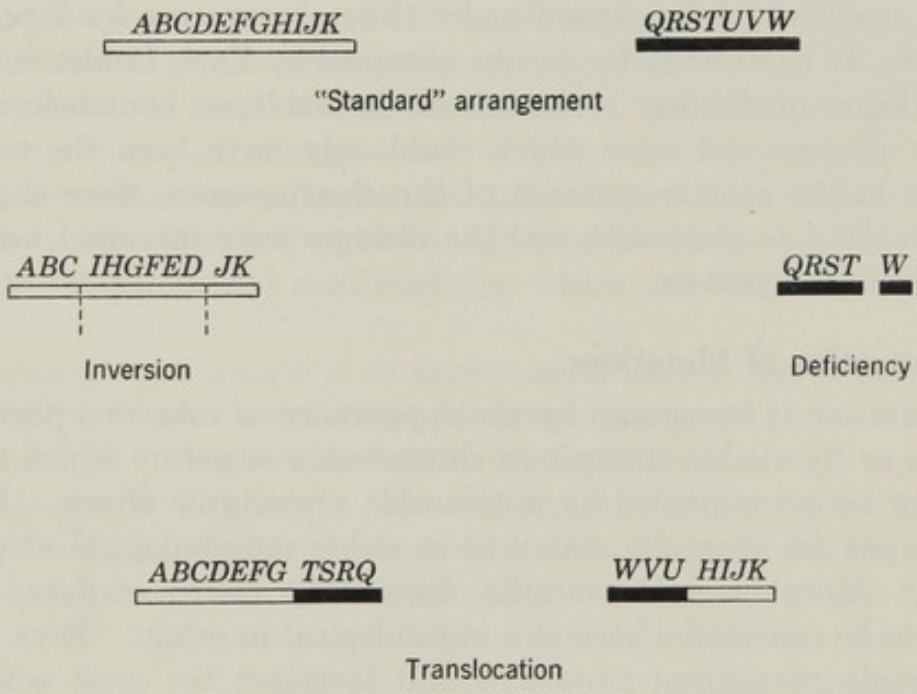


Fig. 12. Types of chromosomal aberrations.

clear material. Mutations in the first class are referred to as *chromosomal mutations,* or *aberrations,* and they may be divided into a number of subgroups, depending on the type of structural change. Some of the different types are illustrated in Fig. 12. Not all these mutations result in detectable phenotypic changes in the organism in which they occur, but it is highly probable that even those which do not are of importance in providing the genetic variability which is potentially capable of manifesting itself as phenotypic variability under the proper conditions. Mutations of the second class, occurring,

as they do, in the absence of any visible alterations in chromosome structure, are considered to be the result of changes at the submicroscopic, chemical level, and are described therefore as *gene mutations* or *point mutations*. By definition, when a gene mutates, it is transformed from one allele to another at that locus.

Some chromosomal mutations are considered to produce phenotypic change as a result of rearrangement of the spatial relationship of genes to one another on the chromosomes. They are therefore arbitrarily described as *extragenic* changes, with the implication that there is not necessarily any modification of the chemical constitution of the chromosome which would in itself result in a new phenotype. They are for this reason distinguished from gene mutations, which are assumed to be *intragenic* in nature.

In *Drosophila*, translocations and inversions frequently result in somatic instability in the expression of certain genes which have been moved to new regions as a result of the rearrangements. This instability is generally expressed as a variegation; that is, some of the cells show mutant characteristics while others around them appear normal. Since it is a variegation resulting from the change in position of genes relative to one another on the chromosomes, the phenomenon has been called *position effect* of the variegated type, (V-type), by Lewis (371). Figure 13 illustrates an inversion in the X-chromosome and a translocation between the X and 4th chromosome which result in variegated type position effects. In the inversion ($N^{264\text{-}52}$) the wild-type genes rst^+, fa^+, dm^+, ec^+, bl^+, and peb^+ have been moved to the right end of the X-chromosome and situated next to the heterochromatin at that end. Five of them produce a variegated phenotype, as a result, while peb^+, the furthest from the heterochromatin, appears unaffected in its expression. Owing to the X-4 translocation, $w^{258\text{-}18}$, the genes w^+ and rst^+ are brought into close proximity to heterochromatin of the left arm of 4, and both of them exhibit an instability in their expression.

In all these examples of variegation it is difficult to determine whether the genes mutate in certain somatic cells to produce the mutant phenotype or their expression is modified by other circumstances. There is a close relationship between the production of variegation and the location of the dislocated gene near the heterochromatin, but it cannot be asserted that location next to the heterochromatin is necessary to produce variegation (231), nor that all genes moved to an abnormal position next to the heterochromatin respond by showing an unstable expression.

Since the rearrangements resulting in position effect are nearly always produced by radiations such as X-rays, there exists the possibility that the relocation of the gene alone is not responsible for the mutant effect, but that the gene's structure has been changed inde-

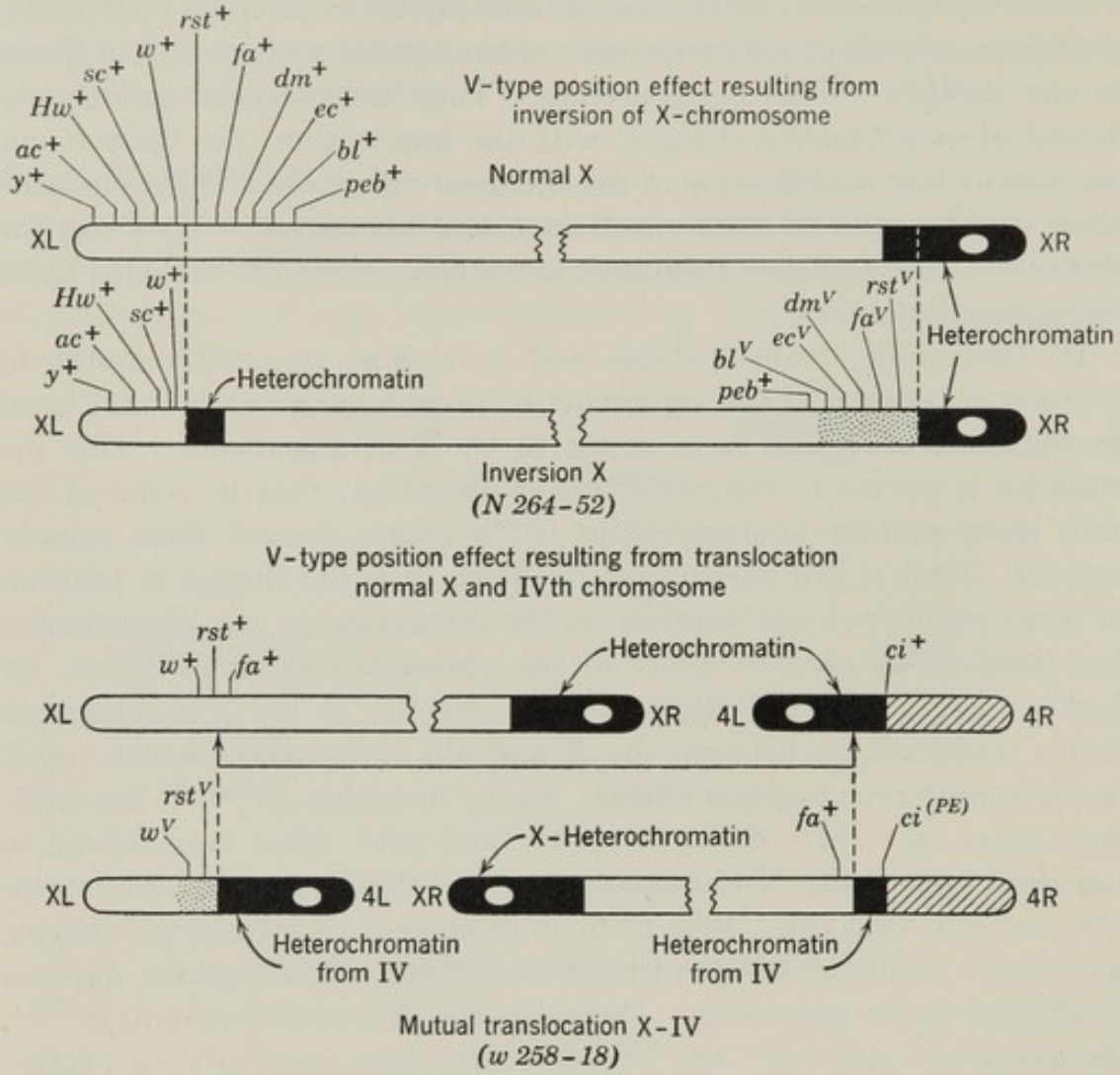


Fig. 13. Types of chromosomal aberrations that result in V-type position effects. After Lewis (371).

pendently of the rearrangement. Proof that this is not so in some position-effect rearrangements at least has been obtained by moving known + alleles into a translocation by crossing over with the production of a V-type position effect (152). Moreover, the position effect may be caused to revert to normal by further rearrangements. Thus the *roughest* phenotype, rst^3, associated with an inversion may be caused to revert to normal rst^+ by reinverting the inverted segment back to the normal position (167, 234). In general, the position effect

disappears if genes showing position effect when abnormally located next to a heterochromatic region are moved by further rearrangement to a euchromatic region (255*a*, 371).

V-type position effect has also been described for the evening primrose, *Oenothera blandina,* by Catcheside (97, 98). This organism and *Drosophila* are the only two in which position effect of this type associated with rearrangements has been proved. However, it is possible that the phenomenon is more widespread than indicated at present, and therefore possibly an important source of variation.

In addition to variegated phenotypes, rearrangements involving inversion and translocation may also produce lethal effects and cause sterility in *Drosophila melanogaster* (448). The rearrangements involving the 4th chromosome (see Fig. 13) frequently have an effect on the expression on the ci^+ gene, which is involved in normal wing venation. When a break is produced in the heterochromatin near ci^+ and a translocation results, the ci^+ gene responds by giving a mutant phenotype in heterozygotes with the mutant allele *ci.* Under normal conditions ci^+ is dominant to *ci* (see Chapter 8).

Cytologically detectable deficiencies in chromosomes invariably produce "visible" mutations or lethality. Some cause a dominant mutant phenotype when heterozygous and act as lethals when homozygous. In a very few cases undoubted deficiencies may be viable homozygous and produce a recessive mutant phenotype (see Chapter 9). Duplications, on the other hand, of single genes or groups of genes may result in lethality or in mutant phenotypes.

Genic versus Chromosomal Mutations

To summarize: (1) a mutation accompanied by phenotypic effects may be *intragenic* (a point or gene mutation), (2) it may be due to obvious structural changes, such as loss or duplication of genetic material, or (3) it may be due to rearrangement of the position of genes resulting in position effect, which may be an *extragenic* change. Actually no method is available for making a satisfactorily clear distinction among these types. This is particularly true for the birds, mammals, fungi, and bacteria used in genetic work, for their chromosomes are generally not satisfactory material for cytological analysis. In organisms such as *Drosophila* and maize, in which certain cells contain chromosomes large enough for detailed microscopic analysis of possible structural changes, the same uncertainty may exist as to whether one is dealing with an intra- or extragenic mutation or deficiency mutation, for many minute rearrangements or losses may lie

well beyond the limits of observation with even these relatively large chromosomes. We can therefore only assume that a mutation may be an intragenic change when there are no visible chromosomal aberrations and when the change is inherited in a simple Mendelian fashion as a single gene. This important point was most ably stated by Stadler (596):

> Mutations in which the altered phenotype is produced by a gene mutation (that is, by the production of a new gene form) cannot be distinguished from these extragenic mutations by any positive criteria. All observed gene mutations therefore are merely presumptive; we can only say that a new allele seems to have arisen, because we cannot detect any of the various extragenic phenomena that might have produced the mutant effect observed.

The significance of this difficulty in establishing the structural basis of mutations inherited as Mendelian factors becomes evident when it is recognized that our knowledge of Mendelian genetics and our recognition of the Mendelian genes are based upon the study of mutations. A gene is recognized only because it has an allelic form which gives a different phenotype. The action of the gene in determining the phenotype is deduced from a comparison of contrasting phenotypes. Experimentally the gene is that portion of the chromosome which undergoes a reduplicatable change or mutation and cannot be separated into cytologically or phenotypically distinguishable parts by crossing over. We can be certain that the mutation process is a chemical one involving the chromosomes. But, if the chemical changes can be either rearrangement, loss, duplication, or just invisible "intragenic" alterations within the transmitted segment, how is it possible to give a precise definition of what we mean by "gene"? This difficulty has led some geneticists, such as Goldschmidt (211), to doubt that genes exist at all. But as stated cogently by Sturtevant (627):

> It is not possible to discuss the questions at issue without using the word "gene" or inventing another term that has the same meaning. There is no escape from the conclusions that chromosomes are regionally differentiated, physiologically as well as visibly under the microscope; that particular and identifiable regions are necessary for particular reactions in the organism, and finally that these particular regions behave as units in heredity—specifically, in crossing over.

2. The Mutation Rate

The study of mutation as a process is ordinarily restricted to (1) considering the types of visible structural rearrangements of the chromosomes, (2) observing changes in phenotype, and (3) determining the

rate at which mutations occur under various conditions. No technique is available as yet to attack the problem at the level of chromosome chemistry, and the experimenter is forced to use the more indirect approaches. One of the more important of these is the study of mutation rate and the factors which influence it, particularly those which increase it over the normal or spontaneous rate.

The mold *Neurospora* has proved to be a valuable organism for the study of mutation rates of specific genes (196, 333). The technique involved is simply to observe the reverse mutation of a mutant gene back to the normal wild-type allele. The reverse mutation rate at a single locus is rather low, but, since the conidia of the mold may be tested in large numbers, i.e., up to several million in a single experiment, it is possible to arrive at a close approximation of the actual mutation rate of the mutant gene to an allele which gives a normal phenotype. Breeding tests can then be performed to confirm the allelism of the gene determining the normal character with the mutant gene.

Since it is not possible to recognize all the mutations that occur in an organism, it is impossible to determine quantitatively the over-all mutation rate. Quantitative measurements may be made by resorting to the method, described above, of measuring the rate of mutation of a single gene, or by determining the rate of occurrence of a class of mutations. Probably the best method for obtaining quantitative estimates of rates for a class are the ClB test or the "Muller-5" technique for detecting the occurrence of lethals on the X-chromosome in *Drosophila* (580). These methods make it possible to determine the number of sperm X-chromosomes bearing recessive lethal mutations quickly and accurately. Although these methods give a very accurate measurement of the rate at which genes on the X-chromosome mutate to a lethal condition, it must be recognized that, as a class, lethals are not homogeneous. They may be the result of point mutations or deficiencies, or gross structural changes and rearrangements which may lead to position effects.

Another method of detecting mutations or changes that may lead to mutation is the direct determination of chromosome breaks and rearrangements by microscopical examination of the chromosomes. By examining a large number of cells, estimates can be made regarding rates. Chromosomal breaks and rearrangements are best observed in organisms having large, easily stained chromosomes, such as *Drosophila,* the grasshopper *Chortophaga,* the spiderwort *Tradescantia,* and maize. *Tradescantia* may be used to determine the number of chromo-

some breaks soon after they occur, with a degree of accuracy not possible with either *Drosophila* or maize. This is because the pollen chromosomes may be observed easily during the stages of nuclear division. The types of chromosomal rearrangements resulting from breaks occurring before or during division may be determined even though they may give rise to nuclei which would be unable to produce viable offspring.

Mutations and Time

It is to be expected that the number of mutations accumulated by a cell will be proportional to the age of the cell. This expectation has been verified by experiments with plant pollen and seeds, and with *Drosophila* sperm. Plant seeds stored for long periods show a large increase in the number of accumulated mutations when compared to unaged seeds (91, 623, 624). Stubbe (624), for example, detected an increase from 1.5% to 14% in recessive mutations in snapdragons, *Antirrhinum majus,* derived from seeds aged from 5 to 10 years. The aging of *Drosophila* sperm in the male or in the sperm storage receptacles of the female results in an increase in the number of lethal mutations on the X-chromosome, as shown in Table 1.

Table 1. Spontaneous Rate of Occurrence of Sex-Linked Lethals in Young and Aged Sperm of *Drosophila melanogaster*

Sperm Not Aged			Sperm Aged 15 to 20 Days			
No. Tested	No. Lethals	% Lethals	No. Tested	No. Lethals	% Lethals	References
9,751 *	10	0.102	8,637	21	0.243	650
13,481 *	14	0.104	18,659	49	0.263	501
3,545 †	5	0.141	3,471	11	0.317	322

* Stored in male.
† Stored in female.

Although these data clearly indicate that mutations accumulate in time, they do not necessarily mean that the time rate of occurrence of mutations is constant. Olenov (459*a*) has shown that *Drosophila* raised on a deficient food medium which increased their developmental period from the normal 10 days obtained on complete medium to 30 days gave sperm with approximately the same number of X-chromosomes with lethal mutations as those with the shorter period of development. The simplest explanation for these facts seems to be that the occurrence of mutations is dependent on physiological factors, and

that mutations are not simply accidents occurring independently of internal environmental factors. Muller (446) and Lamy (337) have reported experimental results in agreement with this hypothesis. They found that the rate of sex-linked mutations in *Drosophila* is different in different stages of the germinal cycle. For example, the sperm accumulated during the preimaginal life of a male show a 2–3 times greater mutation frequency than those produced 6 to 9 days later. In agreement with the results shown in Table 1, Muller did find that the mutation frequency rises considerably in sperm stored in the spermatheca of the female and probably also when stored in the adult male, but this increase is not sufficient to account for the high frequency obtained in the very young males. The X-chromosomes from females when tested for lethals show an almost constant frequency of lethals whatever the age of the parent, which would indicate that mutations occur only in the preimaginal life of the females. Muller (446) concludes therefore that most mutations, particularly in the female, arise at some definite stage in development, such as early cleavage.

It is clear that at least in *Drosophila* the time rate of mutation is not constant in all cells of the individual during its life span, and that it probably varies not only from one stage of development to the next but even within the different stages. It is also evident that the term mutation rate as used by the geneticist is best considered as being the number of mutations per generation, unless the experimental conditions are such that it can be shown that the rate for a specified period during the generation time can be measured.

It is actually very difficult to generalize on this matter at the present time with any degree of confidence, because of conflicting results obtained by different workers. For example, Novick and Szilard (458) have found that in *E. coli* mutation to phage resistance is completely independent of the generation time. In their experiments they showed that under the conditions employed the number of mutations per generation is not constant, but increases proportionately with the absolute time. Thus the mutation rate for a generation time of 12 hours is 6 times and for a generation time of 6 hours 3 times as high as it is for a generation of 2 hours (458). In contrast to these data Zamenoff (721) and others (700) have found a definite correlation between mutation rate and cell division in bacteria.

Spontaneous Mutation

The "normal" mutation rate is that obtained under ordinary conditions of observation without the use of abnormal external agents or

conditions, such as extreme temperatures, high intensities of radiations, and unnatural chemical conditions. It is generally described as being a *spontaneous* rate, although there is no necessary implication that spontaneous mutations occur completely independent of influences outside of the genetic material which undergoes mutation. The spontaneous rate is what one might assume to be an approximation to the mutation rate in any natural population of organisms. It is usually stated to be low, but this needs to be qualified as to whether one is speaking of the rate of mutation of a specific gene or of the total mutation rate of all the genes in the genome. According to Muller (447) the average mutation rate per gene is about 1 in 100,000 to 1 in 1,000,000 in any given cell cycle (generation) in *Drosophila melanogaster*. Thus, for each gene, mutation is a rare event. On the other hand, the over-all rate in this organism is probably about 1 mutation in 10 gametes to 1 in 30, if all genes and all possible mutations (449) are considered.

Spontaneous mutation rates have been determined for a number of specific genes in several organisms, and, although the data are not

Table 2. Spontaneous Mutation Rates at Specific Loci

Organism	Mutation	No. Gametes Tested	Frequency per 10,000	Reference
Maize	$Wx \rightarrow wx$	1,503,744	0.000	593
	$Pr \rightarrow pr$	647,102	0.11	593
	$Sh \rightarrow sh$	2,469,285	0.012	593
	$Su \rightarrow su$	1,678,731	0.024	593
	$I \rightarrow i$	265,391	1.06	593
Columbia stock	$R^r \rightarrow r^r$	20,984	6.2	594
Cornell stock	$R^r \rightarrow r^r$	43,416	18.2	595
D. melanogaster	+ → prune, white, ruby, carmine, singed, raspberry, vermilion, garnet, and forked	60,000	av. = 0.3	451
	+ → cut	60,000	1.5	451
	+ → yellow	70,000	0.29	312
	+ → white	70,000	0.29	312
	+ → lozenge	70,000	0.29	312
Man	Hemophilia		0.32	252
	Chondrodystrophia		0.427	492
	Retinoblastoma		0.23	453*a*
Prunus avium	Incompatibility Genes S_x, etc., to S_y, etc.	60,000	0.003–0.023	368

extensive, they clearly show that not all genes in the same species mutate at the same rate. (See Table 2.) Furthermore, the over-all average rate is different for different species and may vary within different populations of the same species. There seems to be a rather great range of mutabilities, ranging from those genes which are not observed to mutate at all to those which mutate at excessively high rates.

3. Genetic Factors Influencing the Spontaneous Mutation Rate

Laboratory stocks of *Drosophila melanogaster* collected from different sources have long been known to show different rates of mutation (366, 442, 450). Table 3 lists a number of stocks which have been

Table 3. Spontaneous Mutability in Different Stocks of *D. melanogaster* Raised at 22°–25°C

Sex-Linked and 2nd Chromosome Recessive Lethals

	X-Chromosome			2nd Chromosome			
Stock	No. Tested	No. Lethals	% Lethals	No. Tested	No. Lethals	% Lethal	Reference
Florida inbred	2,108	23	1.09	—	—	—	136
Wooster	1,266	8	0.63	—	—	—	136
Oregon R	3,049	2	0.07	—	—	—	136
Florida No. 10	916	10	1.09	516	9	1.74	486
Lausanne	955	2	0.21	436	3	0.69	486
Leningrad	8,614	14	0.16	—	—	—	725
Sukhami	2,309	24	1.04	—	—	—	725

analyzed for lethal mutations, particularly sex-linked recessives. It will be noted that the range for sex-linked recessives runs from as low as 0.07% for the Oregon R strain to as high as 1.09% in the Florida stock. The increase in rate is probably general for all chromosomes, for a high rate in the Florida stock is found for both the X and second chromosomes as compared to the somewhat lower rate in the Lausanne stock. Several of the highly mutating strains have been analyzed in detail. Demerec (136) carried out genetic studies with the Florida stock which revealed that a recessive factor (*mu-F*) on the second chromosome was responsible for the high rate of 1.09% in this strain. Elimination of the factor from the stock resulted in a lethal rate of 0.074%, almost a fifteenfold difference in the strain carrying the factor homozygous. Similar mutator genes have been described in *melano-*

gaster by Neel (452) and Ives (312), and by Mampell (400) in *Drosophila persimilis*. The effect of the mutator genes can be recognized by their production of not only a larger number of lethal mutations but of more visible mutations as well.

Perhaps the most interesting examples of genetic control of the mutation rate have come from the investigations of Rhoades (512, 513) and

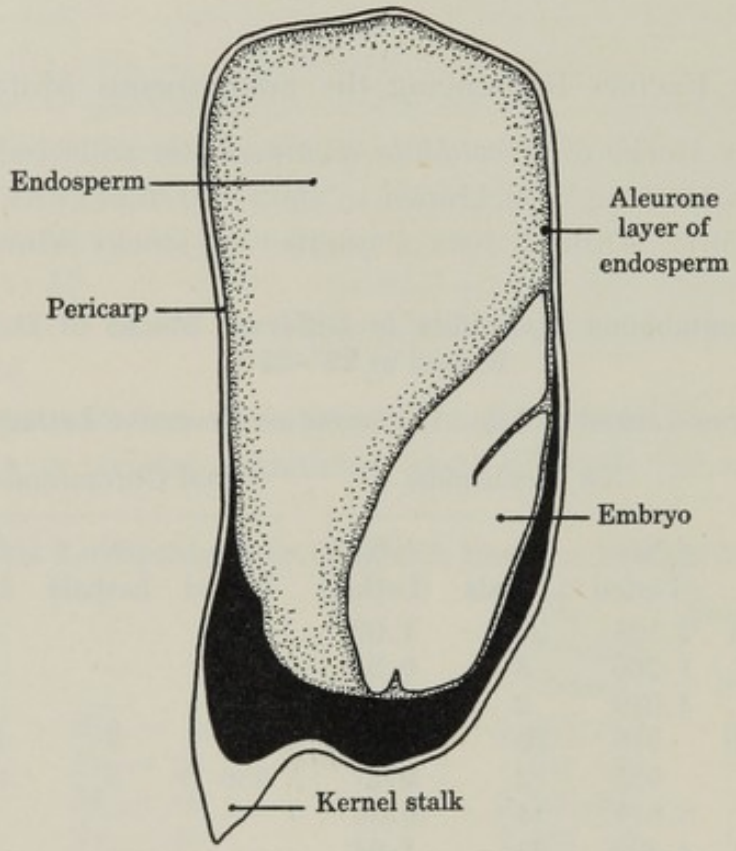


Fig. 14. A diagram of a corn kernel. After Fuller, *The Plant World,* Henry Holt and Co., New York.

McClintock (390, 391) on maize. These studies not only have confirmed the earlier finding from the work on *Drosophila* that the rate of mutation of some genes is under the control of other genes but have also provided further data supplemented by cytological observations which may eventually lead to an understanding of the phenomenon of spontaneous mutation.

Rhoades discovered that the a_1 gene of the A_1 series of alleles in maize, although ordinarily very stable, can be caused to mutate at a high rate to other alleles in the series. This gene is located on the third chromosome, and when it is present homozygous no anthocyan pigment is formed in the aleurone of the endosperm or in the plant (see Fig. 14). However, in the presence of dominant *Dt*, a gene on

the ninth chromosome, it mutates to the other alleles in the A_1 series which are dominant to a_1, and allow the production of anthocyan pigment. Mutation of a_1 occurs both in the germ cells and the somatic tissues. Somatic mutations show up as a variegation of small spots of anthocyan in the aleurone and as narrow stripes of pigment in the plant parts. No other allele in the series is affected by the *Dt* gene in this fashion. The mutation rate of a_1 to the higher members, such as A_1 (which produces the greatest amount of pigment), is most easily measured by observing the appearance of colored spots in the aleurone of genotype *aaa Dt Dt Dt*. During development of the endosperm tissue the a_1 genes mutate to A_1, starting off centers of growth of colored tissue which becomes visible as spots when they get large enough. Each spot is assumed to arise from a single mutation.

The fact that the spots are usually of about the same size shows that the mutation takes place at a definite period of development, and the relatively small size of the spots proves that this period is quite near the end of the development of the aleurone. The same is true in general for the other tissues in the plants, including the sporogenous tissue in the anthers which leads to the production of male gametes. There are interesting dosage effects. As the dosage of the *Dt* allele is increased from *Dt dt dt* to *Dt Dt Dt* in the aleurone, the number of mutations observed per seed increases as follows:

dt dt Dt	gives	7.2 mutations per seed
dt Dt Dt	gives	22.2 mutations per seed
Dt Dt Dt	gives	121.9 mutations per seed

The increase in the number of sensitive *a* alleles in the presence of a constant dosage of *Dt* causes, as would be expected, a linear increase in the appearance of mutations.

4. Physical and Chemical Factors Influencing the Mutation Rate

Besides the considerable influence of the genotype on the mutation rate, there are three major environmental factors which may cause drastic changes in this rate: (1) the temperature, (2) certain radiations and (3) certain chemicals. Factors 2 and 3 in particular have been extensively applied not only in the study of mutation but also to provide a source of new mutations for genetic work. Although

attempts to increase the general level of mutation rate by artificial means have been highly successful, there has been no successful demonstration in sexually reproducing organisms of *directing* the mutation of a specific gene while leaving the remainder of the genetic material unaffected. Mutagenic agents such as radiations and chemicals appear simply to supply enough energy to cause, directly or indirectly, a chemical transformation in the genetic material and thus result in an increase in the mutation rate above the spontaneous level in a random fashion. The experimenter has very little control over the types of mutation obtained by means of this treatment. In general he can control the over-all rate only through variations in the application of the mutagen.

Temperature

The effect of temperature on the mutation rate has been investigated primarily in *D. melanogaster*. As shown in Fig. 15, increasing the temperature at which the fly develops increases the mutation rate,

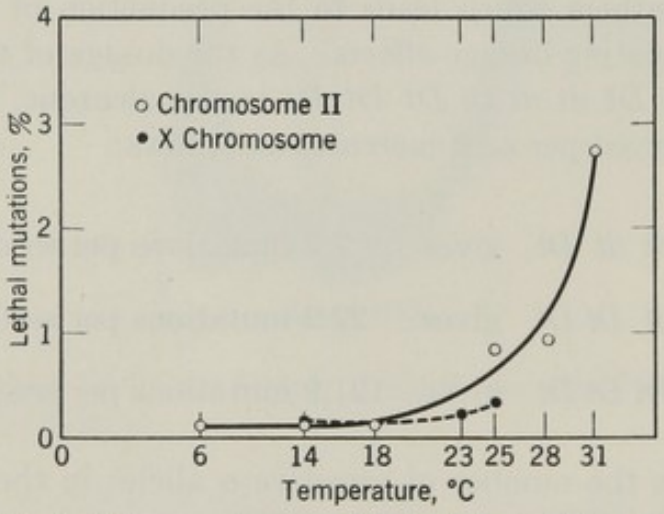


Fig. 15. The effect of temperature on the lethal mutation rate in *Drosophila melanogaster*. From Plough (486).

particularly at temperatures above 15°C. If the generation is used as a biological time unit, the temperature coefficient, $t^{\circ}Q_{10}$ (the ratio of the rate constant at one temperature to the rate constant at a temperature 10°C lower), is calculated from these data to lie between 2 and 3. This is what would be expected for most biological processes and chemical reactions. It is to be recognized, however, that this is an average coefficient for a class of mutations, lethals on the X and second chromosome of *melanogaster*. What the coefficients may be for specific

loci is not known, but it is quite possible that there may be a considerable degree of variance from the indicated mean of 2 to 3.

Temperatures outside the normal range for the organism are not feasible in experiments in which the complete course of development is at a constant temperature, as in the examples described above, but extreme temperatures may be applied for short periods as temperature "shocks." The lethal and visible mutation rate is approximately doubled by treating 3-day-old larvae of *melanogaster* with temperatures of 36° to 38°C for 12 to 24 hours (79, 80). Low temperature shocks of −6°C for 25 to 40 minutes have been reported to triple the lethal rate of mutation for the X and second chromosomes (46). There is no evidence that the mutations produced by the temperature shocks are any different from those occurring under prolonged conditions of moderate temperature, and, like the prolonged temperature treatment, heat shocks seem to have no effect on the translocation frequency (486).

Genes with extremely high mutation rates, the so-called unstable or mutable alleles, seem to respond differently to temperature from the wild-type genes mutating to lethals in *D. melanogaster*. For example, it has been found that the a_1 allele in maize, when in the presence of the specific mutator gene *Dt*, mutates to recognizably different alleles at a rate 4 to 5 times higher at 15.5°C than at 27°C (512). The effect of temperature on the mutation rate of a_1 in homozygous *dt* plants is not known. A similar inverse relation between mutation rate and temperature has been reported for the unstable flaking gene in *Portulaca* (175) and an eversporting eye-color condition in *melanogaster* (221). On the other hand, Demerec (135) was not able to discover any measurable effect of a 10° change in temperature on the mutation rate of several unstable genes in *Drosophila virilis*.

It is clear that no generalizations can be made at the present time based on the known data concerning the effect of temperature on mutation rate. Attempts have been made to reconcile the differences in response shown by the "stable" genes in *Drosophila* to temperature by the application of quantum theory, which dictates a smaller increase in rate of change of unstable molecules with increase in temperature than with stable molecules (448). The theory, however, is not consistent with all the known data. The observed stability of a gene is a function not only of the gene's thermodynamic state but also of its immediate environment in the cell, and it will be necessary for hypotheses attempting to explain changes in mutation rate with temperature to take these environmental conditions into consideration.

Radiation

X-rays, α-rays, neutrons, γ-rays, β-rays, and ultraviolet light are proven mutagenic agents capable of changing the genes and chromosomes. Of these, ultraviolet light is the only non-ionizing radiation. Ionizing radiations are assumed to cause their primary biological effect by producing ionization within the tissue, and to have secondary effects resulting from thermal agitation or excitation of the tissue molecules. It is difficult to assess the relative effectiveness of ionization and excitation (176), although some workers, like Lea and Catcheside (see Lea, 353), have assumed for theoretical purposes that changes due to excitation without ionization are inconsequential. In either case, whether the transformations are caused by ionization or excitation, the molecules hit by a particle or quantum of energy may be expected to undergo chemical change. The probability of the change occurring increases with the amount of energy transferred from the radiant energy particles or quanta. Ultraviolet light, not being an ionizing radiation, produces its effect only by excitation of the molecules of compounds which absorb it. Compounds which do not absorb ultraviolet are of course unaffected by it *directly*, for there is no transfer of energy and thus no cause for molecular agitation which might result in a chemical and hence genetic change. Compounds which do not absorb ultraviolet may, however, be affected by it indirectly, as discussed in succeeding sections.

All these radiations are presumed to exert an effect on genes and chromosomes by providing energy for chemical changes resulting in mutations. The nature of the chemical changes is unknown, but many of them are stable, as shown by the fact that mutant phenotypes produced by irradiation maintain a stability from generation to generation characteristic of naturally occurring mutations. In addition to point mutations, chromosomal breaks are also produced, resulting in inversions, translocations, and deletions, as well as loss of whole chromosomes and other types of abnormal conditions. The extent of change induced in the mutation rate is dependent on the type of radiation, the dose, and various environmental factors discussed below.

The dosage of ionizing radiation applied to a tissue is measured in terms of roentgen (r) units for X-rays and γ-rays. The roentgen unit represents the number of ionizations produced per unit volume of matter irradiated. For water and tissue irradiated by a dose of 1 r, approximately two ionizations are produced per cubic micron, the exact value depending on the type of radiation and the composition

of the tissue. α-Rays, β-rays, and neutrons are radiations emitted as atomic particles. Measuring the dosages of these radiations is more difficult than for X-rays and γ-rays, but they may be converted to r units for comparative purposes, since all produce ionizations.

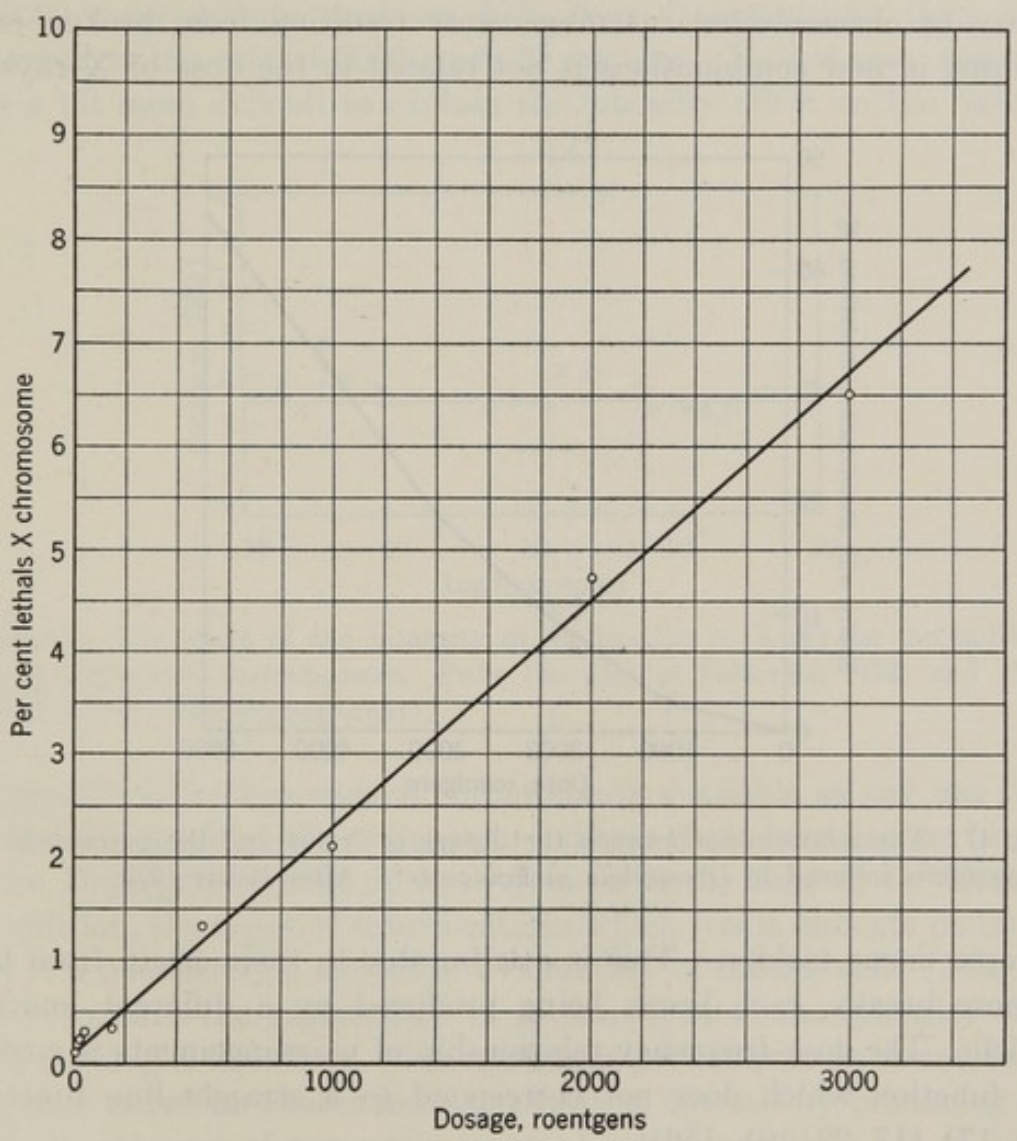


Fig. 16. The relationship between dosage of X-ray and the percentage of X chromosome lethals induced in *Drosophila melanogaster*. Data from Spencer and Stern (580).

There is an apparent direct proportionality between effectiveness of ionizing radiations in inducing "point" mutations and dosage of the applied radiation. This relationship is illustrated in Fig. 16 for sex-linked recessive lethals in *Drosophila melanogaster*. The proportionality has been found to hold for visible mutations as well as for lethals. The number of mutations produced by ultraviolet light is also propor-

tional to dose (ergs/cm^2) but only at low dosages; at sufficiently high dosages the mutation rate fails to increase with increasing dosages or may even fall off.

The frequency of chromosomal breaks produced by ionizing radiations is proportional to the dose (23, 90, 334, 539, 647), but the frequency of chromosomal rearrangements resulting from broken ends rejoining in new combinations is not related to the dose of X-rays in

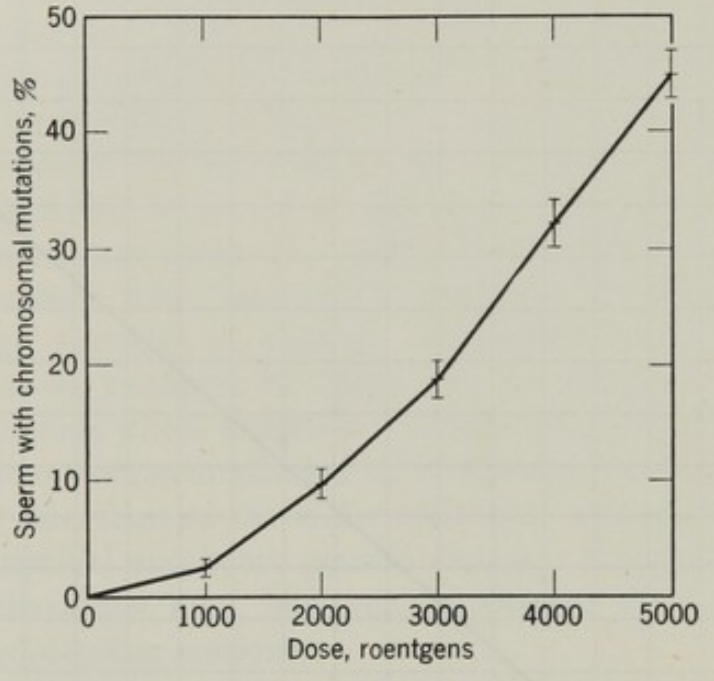


Fig. 17. The relationship between the dosage of X-ray and the percentage rearrangements induced in *Drosophila melanogaster*. After Bauer (22).

a simple linear fashion. This is attributable to their origin from two or more breaks, each break being produced by a different ionizing particle. The dose-frequency relationship of rearrangements is a complex function which does not correspond to a straight-line function (Fig. 17) (17, 22, 101, 156).

The mutation rate induced by ionizing radiations for the most part is independent of the time rate of application of the dose (generally called the intensity) of the radiation (472, 651). Figure 18 gives some data which show that this independence between intensity and mutation rate holds over a very wide range of intensities provided that the total dose is kept constant. There are certain exceptions to this generalization, such as, for example, the production of translocations in *Tradescantia*. Here, as might be expected, spreading the dose causes a reduction in the number of aberrations due to two breaks (540), for

the use of low intensities limits the number of breaks available for forming new unions at any one time.

Haas et al. (243) have found that the production of translocations by X-ray of *Drosophila virilis* sperm is greatly enhanced by the application of very high intensities. The same dose of X-rays delivered at 2000 r per minute produces 60% more translocations than when delivered at the rate of 2000 r per 20 minutes. In this case, however, it is a bit more difficult to explain the intensity effect on the basis of

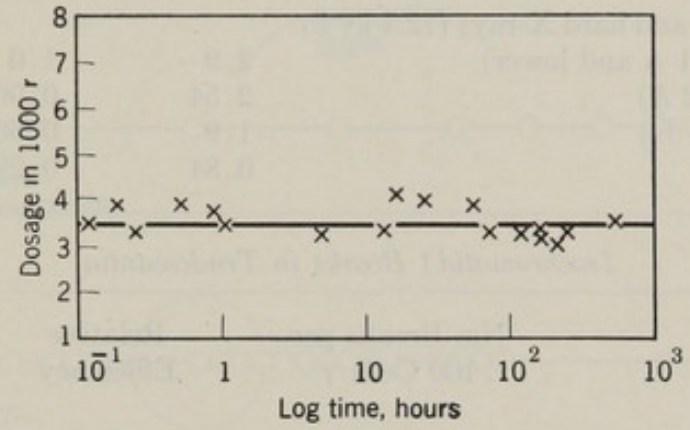


Fig. 18. The effect of the intensity of application of X-ray on the mutation rate of *Drosophila melanogaster*. From the data of Patterson (472) and Timofeeff-Ressovsky and Zimmer (651).

the number of broken ends of chromosomes available at any one time, for there is considerable evidence to indicate that chromosomes broken in the *Drosophila* sperm do not rejoin until the time of fertilization. In addition, the types of translocations which result indicate definitely that it is not merely a matter of availability of broken ends (243). If this is so, then the explanation of at least part of the intensity effect must be sought elsewhere. The answer is probably to be found in the fact that radiation causes indirect effects in the cell by producing free radicals, etc., out of non-genic material, which can then act as mutagens by reacting with the genic material. This indirect effect of radiation and chemical mutagens is discussed at length in the succeeding sections. In the present context it is evident that the concentration of mutagenic material produced by high intensity would be expected to be much greater than with low intensity if the mutagens are labile. This is evidently the situation, as discussed below.

Although all ionizing radiations cause the production of mutations and breaks in chromosomes, they do so with different efficiencies. Table 4 gives some quantitative estimates of the relative efficiencies of

Table 4. The Relative Efficiencies of Various Ionizing Radiations in Producing Lethals in *Drosophila* and Chromosome Breaks in *Tradescantia*

Lethals in Drosophila

Radiation	% Lethals per 1000 r	Relative Efficiency	Reference
X-rays from betatron			
(23 Mev)	1.7	0.59	508
(20 Mev)	1.4	0.48	383
β-Rays, γ-rays, and hard X-rays (12.4 kv to 2.2 Mev) (1 A and lower)	2.9	1.0	723
Soft X-rays (2.2 A)	2.54	0.96 *	723
Neutrons (Li + D)	1.9	0.66	652
α-Rays (radon)	0.84	0.29	677, 353

Isochromatid† Breaks in Tradescantia

Radiation	No. Breaks per 100 Cells/r	Relative Efficiency	Reference
X-rays			
(0.15 A)	0.27	1.0	647
(1.5 A)	0.26	1.0	100
(4.1 A)	0.44	1.6	100
Neutrons (Li + D)	0.99	3.7	647
α-Rays (radon)	2.10	7.8	334
Thermal neutrons	3.02	11.0	114

* Compared to 2.65% lethals at 0.94 A found by these authors.

† Sister chromatids which have presumably been broken simultaneously at the same location along their length during early prophase.

several types of ionizing radiations in producing lethals in *Drosophila* and chromosome (isochromatid) breaks in *Tradescantia*. These data have been collected from several sources, and hence a certain amount of divergence is to be expected as a result of differences in experimental technique. However, the differences shown in the table are so great as to definitely indicate that the ion intensities of ionizing radiations are of some significance in determining their effectiveness. The ion density of a radiation is a measure of the distribution of ionizations produced along the paths of its ionizing particles. Radiations with high ion density such as α-rays, neutrons and soft X-rays have their ionizations spaced close together, whereas the ionizations are more widely spaced in the "hard" radiations, such as short-wave X-rays, β-rays, and γ-rays (see Fig. 19).

The effectiveness of ultraviolet light in inducing mutations is closely related to the wavelength employed. In general, the greatest mutagenic activity is found in the range between 2500 and 2800 A, but the specific wavelength with maximum effectiveness is different for different organisms as shown in Fig. 20. The relative efficiency curve (action spectrum) for maize compares favorably with the absorption spectrum curves of nucleic acids which also have a peak in the region

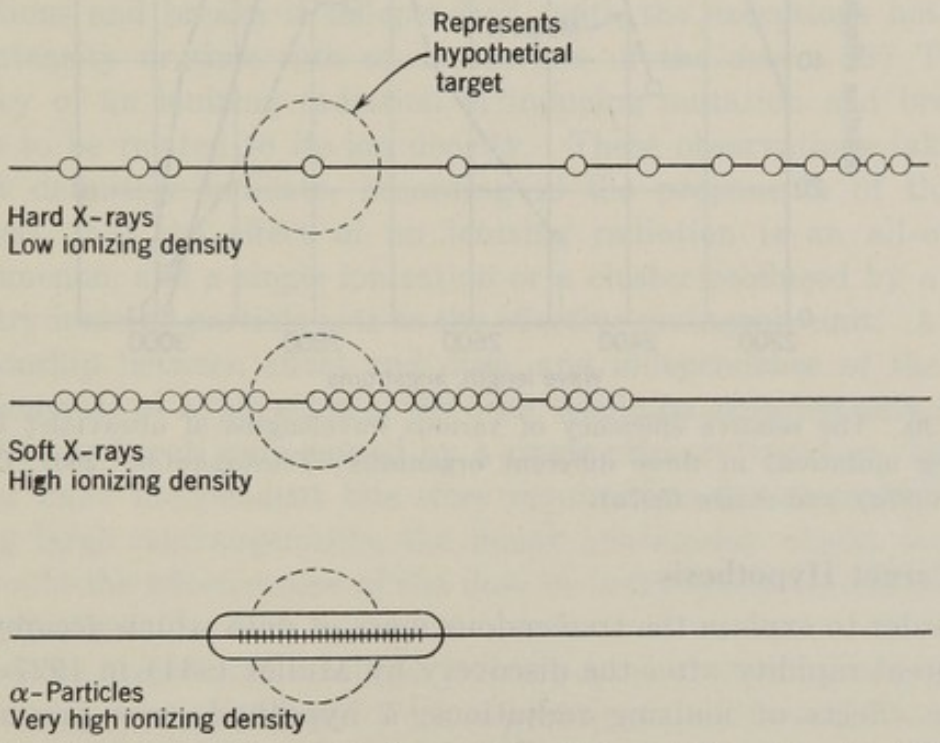


Fig. 19. Diagram of the ionization paths of three different kinds of ionizing radiations. After Gray (224).

of 2600 A. This correspondence has prompted many to suggest that it is positive evidence that nucleic acid is an important functional constituent of the gene (286). Nucleic acid is, as would be expected, rapidly decomposed by disruption of the purine and pyrimidine structure when irradiated by ultraviolet in the region of 2600 A (504). Furthermore, it is well known that the bactericidal activity of ultraviolet is greatest at 2600 A. These facts taken together would indicate some validity for the hypothesis that ultraviolet mutagenic and lethal activity is due to the modification of nucleic acid within the cell. However, not all organisms, as demonstrated in Fig. 20, show the same response to ultraviolet as measured by mutation rate, nor has the possibility of other substances which absorb ultraviolet in the cell being implicated been properly considered in the interpretation of these data.

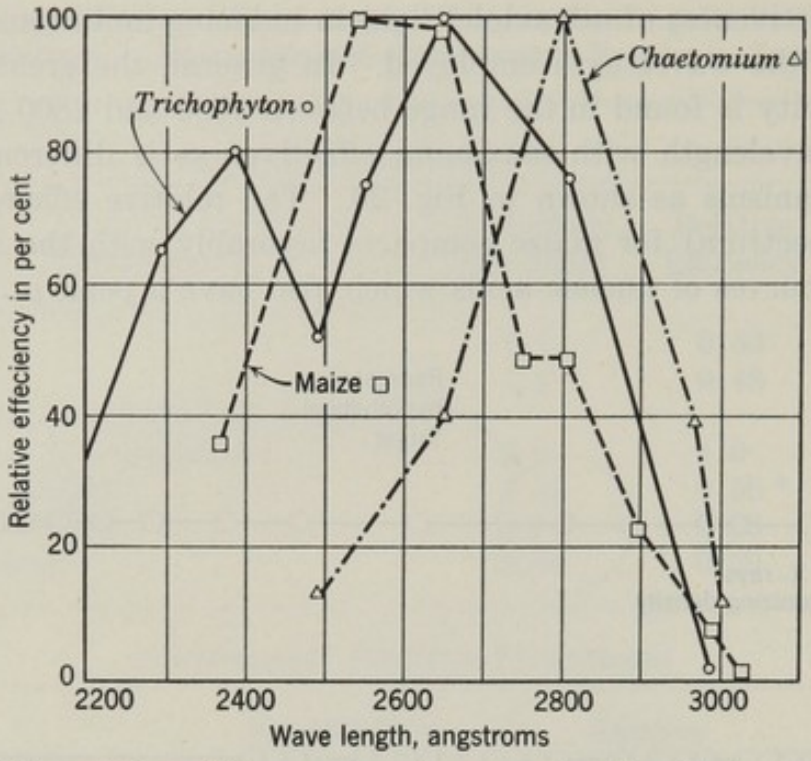


FIG. 20. The relative efficiency of various wavelengths of ultraviolet light in inducing mutations in three different organisms: *Trichophyton* (286), *Chaetomium* (387*a*), and maize (597*a*).

The Target Hypothesis

In order to explain the tremendous mass of data which accumulated with great rapidity after the discovery by Muller (441) in 1927 of the genetic effects of ionizing radiations, a hypothesis was propounded which has been variously described as the direct-effect, hit, target, or Treffer hypothesis. The hypothesis begins with the assumption that simple excitation of the atoms of the irradiated material is of little consequence in inducing chemical change, but that the observed effects are primarily induced by ionization. This is an assumption for which there is no direct supporting evidence from gene mutation studies, and it has been subjected to considerable criticism. See Fano (76) and Gray (224). Next the assumption is made that genes and chromosomes on being "hit" or traversed by an ionizing particle are caused to mutate with a probability close to 1. Though acknowledging that ionizing radiations cause molecules in the cell other than the chromosome molecules to undergo transformation, the hypothesis in its purest form does not concede that the altered extragenic material will in turn affect the chromosome constituents to any significant degree (353). To allow for the possibility that an ionization very near the chromosome may have an indirect effect on the chromosome, it is generally assumed that

there exists a sensitive volume which includes the chromosomes. An ionization must occur within the sensitive volume to produce a point mutation or a chromosome break. Therefore the target is the sensitive volume, and hits on the target may be detected as mutations.

The target hypothesis is mainly supported by the following evidence. (1) The number of induced "point" mutations or chromosome breaks observed appears to be directly proportional to the dose as determined by the number of ionizations measured. (2) The yield of "point" mutations and breaks is independent (with the exceptions noted) of the intensity or time rate of application of the dose. (3) The efficiency of an ionizing radiation in inducing mutation and breakage seems to be related to its ion density. These observations taken together definitely indicate, according to the proponents of this hypothesis, that the effect of an ionizing radiation is an all-or-none phenomenon, and a single ionization or a cluster produced by a single primary ionizing particle acts as the effective mutagenic unit. A linear relationship between effect and dose, and independence of the effect and time rate of application of the dose, would be expected only if each mutation or break were caused by a single primary event or "hit." If two or more independent hits were required, as for example in producing large rearrangements, the linear relationship would not hold, nor would the effectiveness of the dose be independent of the intensity of application.

That the "hits" may be single ionizations or clusters is indicated by the data given in Table 4. With reference to isochromatid breaks in *Tradescantia,* it will be noted that these are produced most efficiently by α-rays. Neutrons, very soft X-rays, and hard X-rays follow in effectiveness in decreasing order of efficiency. This corresponds to the order of decreasing ionization density. It has been concluded that the direct relationship between decreasing efficiency and decreasing ionization density indicates that the breaks in *Tradescantia* chromosomes are produced by a number of ionizations in a group (354) and not by single ionizations. The ionizations produced by hard X-rays are so spread out along most of the track that only the tail ends are effective, whereas most or all of the track range of α-particles and protons produced by neutrons would be effective. (See Fig. 19.) Lea and Catcheside (354) have calculated from data such as these that the minimum number of ionizations necessary to break a *Tradescantia* chromosome lies between 15 and 20.

The efficiency of X-rays, neutrons, and α-particles in inducing lethals in *Drosophila* decreases with increasing ion density. Lea (353) and

Catcheside (99) have interpreted this as meaning that recessive lethals in *Drosophila* are produced by single or at most a very few ionizations. The bombardment of a small object, such as the gene is assumed here to be, with ionizing particles from α-rays, producing ionizations spaced so closely together along the track that many occur almost simultaneously within the target or sensitive volume, would result in some of the ionizations being wasted, if only one is necessary to produce the effect. (See Fig. 19.) On the other hand, with radiations of lower density, the efficiency would be increased, since the ionizations are more widely distributed, and each will have a higher probability of making a "hit" in a different location and hence produce a different mutation. It will be noted that the extremely high-energy X-rays produced by the betatron do not give results which conform to this interpretation, despite the fact that they have the lowest ion density among the sources listed in Table 4.

Proceeding on the assumption that the target hypothesis is correct, and that a gene mutation may be produced by a single ionization (or an ion cluster with secondary ionizations produced by the electron projected by the primary ionizing particle), numerous workers have attempted to estimate the size of the gene from radiation data. The linear relationship between dose and number of observed mutations may be stated in the equation

$$n = \alpha ND$$

in which n is the number of observed mutations induced in N chromosomes with a radiation dose D. The proportionality constant, α, may be defined as the probability that a gene will mutate when exposed to a dose of 1 r. Since the probability of a gene's mutating under a barrage of ionizing particles will be directly related to its volume, or the volume of the "sensitive" area around the gene, the volume of the gene may be calculated by means of the above formula. Assuming that genes are spherical, Lea (353) has estimated their diameter by this method to lie between 4 and 6 mμ. Some support for the application of the target hypothesis to the estimation of particle size is to be found in the results from the inactivation of virus particles by ionizing radiations. The dysentery phage S13, for example, has been calculated by the use of γ-rays, X-rays, and α-rays to have a diameter of about 16 mμ (353). This is also the observed diameter of the virus in the unhydrated state as determined by filtration through collodion membranes.

Despite the fair degree of correspondence between the target hypothesis and some of the observed facts, it is becoming increasingly

clear that its use for the interpretation of radiation data in genetics is in need of considerable revision, for, while it has been useful as a working hypothesis, results from investigations to be discussed in the succeeding paragraphs make evident the fact that it may well be incorporated into a more general theory in the future. The criticisms which may be leveled against it are primarily concerned with its use as a means of estimating the size of genetic units. They may be stated as follows: (1) There is no assurance that every hit within the gene or chromosome or its "sensitive" volume produces a mutation. (2) There is considerable evidence that the "sensitive" volume does not correspond to the volume of the genetic material. In other words, the effect of the initial ionizations may be quite indirect in producing mutations in the chromosomes. (3) It cannot be expected that all mutations induced by ionizing radiations can be detected by the present methods. (4) The measurement of radiation dosage only by the ionizations produced results in the factor of excitation of the molecules within the cell being neglected. There is no assurance that excitation by ionizing radiations does not also cause point mutations and chromosome breaks just as occurs for ultraviolet light.

Chemical Mutagens

The possibility of producing mutations by chemical treatment had been extensively explored by numerous workers before and after the discovery of the mutagenic action of radiation. However, it was not until the work of Auerbach and Robson (11, 12), begun in 1940, that it was clearly established that chemical treatment could be as effective as radiations in inducing mutations. The original results were obtained by treating *Drosophila melanogaster* with allyl isothiocyanate (mustard oil), or mustard gas, and then testing for sex-linked lethals. With mustard gas it was found possible to increase the percentage of sex-linked lethals from about 0.2% in the controls to as high as 24% in the treated flies (11). Subsequent work by these and other workers on *Drosophila,* certain of the higher plants, fungi, and bacteria soon made it apparent that many other chemicals besides the mustard vesicants are mutagenic. Table 5 lists some of these compounds and indicates their effectiveness in inducing mutations in *Drosophila,* bacteria, and *Neurospora,* and cytologically demonstrable changes in plant chromosomes, resulting from breaks.

Obviously, no general chemical relationship exists among the compounds listed in Table 5. The list could be extended several times its length as given, by adding compounds which are known to be muta-

Table 5.* Compounds Which Increase the Mutation Rate and Cause Breaks in Chromosomes

Mutagen	*Drosophila*	Plant Chromosomes	Bacteria	*Neurospora*
Mustards †	+	+	+	+
Hydrogen peroxide	?	−	+	+
Organic peroxides	?	+	?	+
Formaldehyde	+	+	+	+
Diazomethane	+	−	?	+
β-Propiolactone	?	+	?	+
Caffeine	?	+	+	+
Urethane	+	+	+	−
Phenols	+	+	+	−
Ethyleneimine	+	+	?	+
Ketene	+	?	?	−
Epoxides and diepoxides	+	+	?	+
Penicillin	?	+	?	?
Carcinogens	?	?	+	−
Na desoxycholate	?	?	+	−
Acriflavine	?	+	+	?
Ferrous chloride	−	?	+	?
Manganous chloride	?	?	+	?
Aluminum chloride	?	+	?	?

* Data from Jensen, Kirk, Kölmark, and Westergaard (316), with additions.
† Both sulfur and nitrogen mustards.

genic for bacteria and to cause chromosome aberrations in plants. To do so would only add to the diversity of chemical structures already represented in the table, which ranges from simple inorganic compounds to complex alkaloids.

A compound may be mutagenic for one organism and have no effect on another. Thus hydrogen peroxide and diazomethane cause mutations in *Neurospora* but produce no evident effect on plant chromosomes. On the other hand, urethane, phenols, etc., produce mutations in *Drosophila* and chromosome aberrations in plants, but no reversion mutations in *Neurospora*. The reasons for these differential effects remain to be elucidated. The explanation may well be that some chemical mutagens are active only during a particular stage of the development of the germ cells or their ancestors. For example, formaldehyde seems to act mutagenically only at an early stage of the developing sperm of *Drosophila* which Auerbach (10) calls the "sensitive" stage. Furthermore, only the male gametes are affected. The treatment of female larvae or adults with formaldehyde has no effect whatever on the frequency of lethal mutations in the eggs (10, 275). This

is to be contrasted to the action of urethane, which is effective on the mature sperm of *Drosophila* but has no apparent effect on the spermatogonia.

Since usually only one stage or part of the life cycle is treated in experiments testing chemical mutagens, it is quite possible that the stage of an organism sensitive to a particular chemical may not have been the one treated, thus giving negative results which might have been positive had the proper stage been treated. One must also assume that the ability of these compounds to penetrate into the cells is different from one organism to another, or that the compounds are broken down by active enzymes before they are able to produce mutations. Negative results should not therefore be taken as final evidence that the substance tested is not mutagenic without thorough testing under different environmental conditions and at different stages of the life cycle.

The mustards, urethane, and formaldehyde all produce the same types of mutations as ionizing radiations in *D. melanogaster* (10). Both biochemical mutations and morphological mutations as well as reversions to wild type are induced in *Neurospora* by mustards and hydrogen peroxide. Doubtless many of the other active mutagens also are as effective in producing the complete spectrum of mutant types, but further testing will be necessary to determine this. Despite the fact that mustards induce all the mutations characteristic of ionizing radiation, however, there are certain differences in their effect from those of X-rays. The frequency of translocations and large deletions is much lower than for X-rays. Thus Auerbach and Robson (11) found that "equivalent" doses of X-rays and mustard gas, as determined by the number of lethals, gave 30% and 6.7% lethal translocations, respectively, and about twice as many large deletions are produced by X-rays as by mustard (11). Another significant difference between the effects of X-rays and mustard gas is that mustard gas seems to have delayed effect in *Drosophila* on the production of mutations. It may produce a mutation in a cell a generation or more removed from the treated cell (10). X-rays do not cause a similar delayed effect detectable by the techniques employed.

As a result of the discovery of chemical mutagens, a new point of view has evolved with respect to mutation. The genetic material had during the 1930's been looked upon, almost mystically, as being intrinsically exceptionally stable, and subject to chemical modification only by the use of extremely large amounts of energy carried directly

to the gene and chromosomes by penetrating radiations. It is now necessary to recognize that it may be no more stable than any chemical compound. It is apparent that the previously assumed extreme stability of the genetic material is a result of the protection afforded by the non-genic material surrounding it. Indeed it should not have been surprising to find that the genetic material reacts chemically and is changed thereby, for the fact that mutation is under genetic control is in itself strong evidence, if not proof, that extragenic chemicals in the cell can bring about mutation. Otherwise there would be no rational explanation for the phenomenon of one gene having an effect on the mutation of another.

This view is strengthened particularly by the observation that naturally occurring compounds such as allyl isothiocyanate, hydrogen peroxide, formaldehyde, and various purines are effective mutagens. Indirect evidence that hydrogen peroxide produced during the course of aerobic respiration may be a factor in determining part of the spontaneous mutation rate is provided by the results of experiments with catalase and inhibitors of catalase (316, 714). If catalase is added together with hydrogen peroxide in the treatment of *Neurospora* conidia or bacteria, the mutagenic activity of the peroxide is stopped, presumably because the H_2O_2 is destroyed by catalase. If catalase poisons such as KCN or sodium azide are added, the mutation rate increases even in the absence of added peroxide, presumably because the hydrogen peroxide synthesized by the organism accumulates instead of being decomposed by the cell's own catalase. The addition of catalase poisons together with hydrogen peroxide, as would be expected, increases the mutagenic activity manyfold in *Neurospora.*

It is evident that the living cell possibly provides for its own mutations by means of certain of its metabolic products reacting with its genic material. In addition, there may also be found here an explanation for the observations described above that mutations may occur more frequently at one stage of the life cycle than at others, or that there is a relation between the physiological state and mutation rate. As differentiation proceeds, one would expect metabolic conditions to change within the cells and tissues, and hence mutagenic chemicals may be produced in effective concentrations at certain stages but not at others.

Environmental Effects on Action of Mutagens

The action of mutagenic radiations and chemicals in inducing mutations is not independent of environmental conditions at the time of

treatment. Some of the factors which affect the mutagenicity of chemicals have already been noted. The effectiveness of both ionizing radiations and ultraviolet light in producing "point" mutations and chromosome aberrations is considerably altered by the physiological state of the cell being irradiated. Table 6 lists a few of the many

Table 6. Factors That Influence the Induction of Mutations by X-Ray

Modifying Factor	Effect on Mutation	Reference
Absence of *oxygen*, or less than 20% oxygen during irradiation	Greatly reduced induction of sex-linked lethals and translocations in *Drosophila*, mutations in bacteria, and chromosome aberrations in *Tradescantia*, *Vicia*, and maize	6, 18, 198, 243, 553, 648
Carbon monoxide in the absence of oxygen or in presence of low concentrations of oxygen	Increase in the frequency of chromosome aberrations in *Tradescantia* and translocations in *Drosophila*	243, 329
KCN at concentrations of 10^{-3} to 10^{-4} *M*	Increase in the mutation rate of visibles in barley	128
Temperature of 4°C or less during irradiation	Significantly higher frequency of chromosome aberrations in *Tradescantia* and lethals and translocations in *Drosophila* than at 20 to 30°C	17, 328, 413, 541
Pre- and post-irradiation treatment with *infrared*	Increase in chromosome aberrations in *Tradescantia*	631
Degree of *hydration* of seed under irradiation	Mutation frequency of barley increases with increased hydration	320
Stage of development of cell, phase of cell division, etc.	Mature sperm of *Drosophila* more sensitive than spermatogonia; late prophase and metaphase chromosomes of *Trillium* more easily broken	448, 577

known factors which influence the mutagenicity of X-rays presumably by changing the physiological state of the cells in such a way as to modify the number of observable mutations produced.

There may be a number of reasons why these factors cause a change in the observed rate. (1) They may be effective in increasing or decreasing the rate independently of X-rays. (2) They may modify the cell's metabolism in such a way that the cell recovers more easily from "extragenic" radiation effects and hence more mutations are observed. (3) They may predispose the genes to the action of X-rays

by getting them into a labile state. (4) They may create conditions within the cytoplasm and other extragenic materials which allow for the production of compounds which when irradiated form mutagenic chemicals. (5) The gene may be placed in a labile, intermediate "semistable" state by the action of the mutagen, from which state it may revert to the stable original state or proceed to a stable mutant state (393). The influence of the cell environment might be expected to be considerable in determining in which direction the semistable gene may go, and hence it may have a considerable effect on the mutation rate (393).

The first possibility has been eliminated as an explanation for effects given in Table 6 by the use of the proper controls, even though some of these factors have an independent effect on the mutation rate. The last four hypotheses are all, to one degree or another, possible explanations for the observed phenomenon; at least, no one of them has been completely eliminated. But, in any case, it is now firmly established by such data as these that the effect of X-rays and perhaps other ionizing radiations may be far from being a purely physical phenomenon involving only direct ionization or excitation of the genic material with no influence of the extragenic environment. The direct effect of radiation quanta or particles is still probable, but to this direct effect there must be added the indirect effects indicated, particularly the effects of oxygen and carbon monoxide on the mutation rate.

The indirect effect of radiation implied by the observations discussed above receives direct proof for ultraviolet light from experiments performed by Stone, Wyss, and colleagues. These workers were able to demonstrate that mutations in the bacterium *Staphylococcus aureus* are induced by placing the cells into a nutrient medium previously irradiated with ultraviolet light (614, 615). Mutations resulting in resistance to streptomycin and penicillin as well as inability to ferment mannitol were found to occur at a rate of 10 to 500 times higher than the spontaneous rate. Irradiation of the medium instead of the cells also produces mutations in *Neurospora,* although not at such a high rate as detected in *S. aureus* (675). It appears very likely that the mutagenic agent produced by the irradiation of the nutrient medium is hydrogen peroxide or perhaps some organic peroxides (675, 714). As noted in Table 5, peroxides are mutagenic. Although similar experiments have not as yet been reported with X-ray or other ionizing radiations, it is highly probable that similar direct evidence of indirect effects will be obtained, since these, as well as ultraviolet light, are known to produce hydrogen peroxide, the free radicals, OH, H, HO_2,

and probably other peroxides and free radicals in aqueous solutions (15, 20, 681).

The demonstration of an indirect effect for radiations in producing mutations brings their mutagenic effect into the realm of chemical mutagenesis. Although it is hardly likely that radiations act only through the medium of chemical mutagens in producing chromosomal and genic changes, it does seem probable that a considerable part of their activity is expressed in this indirect fashion. Since there is no method of determining quantitatively the direct and indirect effects of radiations in producing mutations, the target hypothesis must be regarded as highly approximate in its application to the interpretation of radiogenetic data.

5. Transformation in Bacteria

The genotype and phenotype of certain strains of bacteria can be transformed by subjecting strains of one genotype to the influence of extracts from cells of a different genotype. Superficially this would appear to be equivalent to mutation, and it has been described as such by some (48). But, as will be made clear below, the phenomenon is not mutation in the sense that the term has been used previously in this chapter. It is, therefore, better described by a less specific term—transformation. It was first discovered in *Pneumococcus* by Griffith (232) and later elaborated upon and more definitely characterized by Avery, MacLeod, and McCarty (14) and Ephrussi-Taylor (173).

Most *Pneumococcus* strains consist of two forms or types: (1) a virulent, smooth (*S*) type which possesses a polysaccharide capsule with specific antigenic properties and (2) a non-capsulated rough (*R*) form which has neither the virulence nor the serological specificity of the *S* form. The two types can readily be distinguished macroscopically by the difference in colony morphology and microscopically by the presence or absence of the capsule.

The different strains of *S* type *Pneumococcus* are characterized by differences in serological specificity inherent in the nature of their polysaccharide capsules. Thus there is a series of *S* strains, designated as *S*II, *S*III, etc., differentiated by the types of antibodies produced by the polysaccharide antigens. The specificity of a given *S* strain is stable, and direct mutations from one *S* type to another, *S*II $\rightarrow$ *S*III, for example, have never been observed. *S* types will, however, mutate spontaneously to *R* type. The *R* types so obtained do not spontaneously revert to *S*, but, if placed in a medium containing a cell-free

extract of *S* cells and certain serum factors including anti-*R* antibodies, they will transform to *S* cells of the same serological specificity as the *S* cells from which the extract is derived. This transformation is permanent in so far as the *S* cells will reproduce more *S* cells and continue to produce polysaccharide capsules so long as mutation to the *R* form does not occur.

The active factor present in the cell extracts from *S* cells has been highly purified and shown to be desoxyribonucleic acid (DNA) (388). As little as 1 part in 600,000,000 of purified DNA extracted from *Pneumococcus* of type *S*III is all that is needed to effect the transformation of *R* cells obtained by spontaneous mutation from cells of type *S*II (Fig. 21).

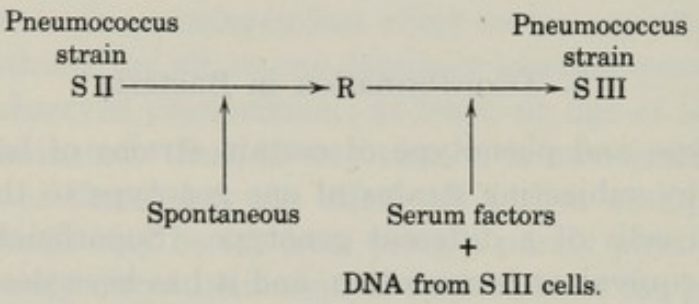


Fig. 21. Transformation in *Pneumococcus*.

Transformations have not been restricted to capsular changes in *Pneumococcus*. Protein specificity (13), penicillin resistance (301), and colony morphology (173) have been some of the characteristics used to demonstrate transformation both by the use of crude cell extracts and purified DNA preparations. Hotchkiss (301) was able by the use of DNA extracts from *S* (smooth) cells possessing resistance to penicillin to show that the transforming principle from these cells had the capacity to transform *R* (rough) penicillin-sensitive cells to the *S*, penicillin-sensitive, or *R*, penicillin-resistant, or *S*, penicillin-resistant conditions. The DNA extract, then, contains at least two principles, one for capsule formation, another for penicillin resistance. Presumably DNA extracts contain a multiplicity of transforming principles.

Transformations have been obtained with *Hemophilus influenzae* (4), and probably in *Escherichia coli* (48, 49), and *Shigella paradysenteriae* (679), using approximately the same techniques as described for *Pneumococcus*. Transformation is therefore not to be considered a phenomenon unique to *Pneumococcus* but rather one with general significance with respect to genetic change in bacteria.

The simplest explanation of the transformation phenomenon is not that it is a directed mutation in which a specific type of DNA effects

a change in the hereditary units or genes of the cells to be transformed but rather that the transforming principle in the form of DNA enters the cell and becomes part of its genotype. Transformation is in this sense a matter of *addition* to the genotype rather than a *change* in the existent genotype. Evidence obtained by Ephrussi-Taylor (173) in studying transformation of colony morphology in *Pneumococcus* would indicate a closer relationship to the formation of recombinations by crossing over found in the higher organisms. Hence transformation is to be looked upon as the result of a type of sexual reproduction in which a genetic element is introduced vicariously as an extract rather than intact as part of another nucleus by fertilization.

6. General Comments on the Nature of Mutation and the Genetic Material

The answer to the question of the relation between gene mutations and chromosomal mutation or aberrations is fundamental to our understanding of the mechanism of mutation. It is a problem which has received considerable attention from geneticists, some maintaining that gene mutations and chromosome mutations are distinctly different, others that gene mutations are simply small, invisible aberrations. As stated previously there is no way of proving the contentions of either side, but there is considerable circumstantial evidence to support the views of those who maintain that a distinction does exist.

First, not all gene mutations can be due to loss of genetic material, for it has been shown repeatedly that reversals occur from the mutant to normal allele and back again to the mutant (195, 319, 333, 473). It would not seem likely that the mutagenic agents used to effect the reversal have the capacity to cause a particular gene to arise *de novo* where one had not existed previously.

Second, the differential effect of the various types of mutagens would in itself indicate some difference in origin of mutations. As pointed out above, ultraviolet light, ionizing radiations, and chemicals such as mustard gas differ in their relative efficiency in producing chromosome aberrations versus gene mutations. In corn, according to Stadler and Roman (597), X-rays produce aberrations but no demonstrable gene mutations. The ratio of aberrations and gene mutations produced in barley seedlings irradiated with X-rays is greatly affected by heat shocks applied before or after irradiation (85). When the combination treatment of heat and X-ray is given the seeds, the number of

chromosome aberrations recovered is reduced 30 to 40% and the gene mutations increased a like amount or more.

Third, from the evolutionary standpoint, the loss of genetic material, or its rearrangement without accompanying gene mutation, could not be expected to produce the high degree of genetic diversity which exists in the organic world at present. Even though loss and rearrangement are contributing factors to genetic variation, it is obvious that they are fairly limited in their contributions. Furthermore, they do not provide the necessary explanation for the increase in the amount of genetic material which must occur during the evolutionary process (445).

Perhaps the present consensus of opinion on this matter among geneticists can be said to center around the following statement as a mean. Gene mutations are the result of undefined chemical changes in the chromosome which are restricted to the portion of the chromosome at which the mutated gene has its locus. Visible chromosome changes and some invisible ones may result in mutation due to (1) loss of genetic material as in deficiencies, (2) increase as in duplication, or (3) rearrangement of genetic material as in translocations and inversions. Whether the chromosomes are viewed as macromolecules or groups of macromolecules, in either case it can be said that chemical changes have occurred as a result of these aberrations. If it is preferred, they may be called "architectural changes" (210). But, whatever term is used, very little is added to our knowledge of what mutations are, beyond recognizing that some are visible and some invisible at the chromosome level, and all can be duplicated in the process of cell division.

Mutation, then, is probably best considered as a chemical change in the genetic material. From the experiments using mutagens it is evident that it is a process requiring energy from the outside. Even in those cases alluded to as "spontaneous" the present evidence indicates that chemical changes within the cell can provide the necessary energy through normal chemical metabolites assuming the role of mutagens. Genes must be complex molecules or aggregates of complex molecules. These molecules almost certainly can exist in many isomeric spatial configurations and carry a great variety of chemically functional groups that contribute to the total complex processes that occur in association with gene action. Some of the functional groups that might be expected are —OH, —SH, —COOH, —NH_2, $\rangle$C=O, —H, and various combinations of these, such as are found in peptide, ester, and

hemiacetal linkages. Each of these, as well as other groups such as alicyclic and heterocyclic biochemical constituents, is especially reactive toward particular chemical reagents. The resulting reactions can result in the formation of new chemical bonds or in the splitting of bonds that already exist. Thus it is quite conceivable that any chemical reaction with genic material might change the pattern of the processes of gene action and result in the production of a reproducible change in the spatial configuration of a macromolecule. If mutation can occur in this manner, then any chemical substance will act as a mutagen, if it can penetrate the cell to the proper place at the proper time and react with one or more functional groups of the genetic material. The mechanism of action of radiations in producing mutations could be similar, but, on the other hand, radiations may also produce their effects without intervention by reactive chemical intermediates. It seems best to consider that mutation can occur in both ways and by combinations of the two.

Of course, it must be recognized that the genetic material, like any chemical substance, is subject to spontaneous changes due to the energy within the molecules themselves and according to the laws of quantum mechanics. See Schrödinger (548) for an interesting discussion of this. However, the spontaneous mutation rate resulting from chance perturbations within the gene molecules must be taken as the "base" spontaneous rate, or the "true" spontaneous rate. Each gene must have its own degree of instability which subjects it to spontaneous change at a certain rate. "Spontaneous" changes above this must be assumed to be due to perturbations in the environment surrounding the gene; otherwise there would be no explanation for the same gene in different environments showing a different spontaneous mutation rate. It is because of the presence of "natural" mutagens that only extremely tentative conclusions can be drawn from the data relating to the effects of temperature on the spontaneous mutation rate. While increasing the temperature would be expected to raise the energy level of the genetic material and thus cause it to mutate more often, the temperature at the same time must have an effect on the production and action of natural mutagens within the cell. What this effect is, is not known. Presumably it is very complicated, for many substances and reactions must be involved. This is particularly borne out by the observation that either cold or hot temperature shocks can cause mutation, a fact not in harmony with the purely physical interpretation.

Relatively few conclusions about the nature of the genetic material can be drawn from the enormous mass of experimental data relating

to the process of mutation. The wealth of genetic variety that has been collected as the result of inducing mutations artificially has been of incalculable value in providing the geneticist with tools with which to work but has given him only a meager understanding of the tools themselves.

Rather than risk the charge of maintaining a hopeless outlook on this particular aspect of genetics, we must stress the amount of information which has been the reward of the mutation investigations carried on thus far. In brief, it may be stated as follows. (1) Significant genetic material is located on the chromosomes or is the chromosomes. Of the many thousands of mutations that have been observed genetically, only a small percentage have been found to be inherited extrachromosomally. (2) The chromosomes are seriated into units of action, the genes, which, although not independent in their action of other genes in producing the phenotype, nonetheless appear to exist as physical and biological entities. (3) Genes may exist in different states, i.e., alleles, which are presumably related to differences in chemical states. No observable cytological change is produced in the chromosomes as the result of these changes in state. (4) The arrangement of genes on the chromosomes is an important factor in determining their phenotypic expression. (5) The high degree of stability of most genes indicates that they are chemical substances which exist in a relatively protected environment. Each gene must have a degree of inherent stability, but outside of the cell it undoubtedly decomposes in the unnatural environment. Despite their buffered condition, however, genes undergo changes "spontaneously," probably most often under the influence of the environment, both chemical and physical. The "spontaneous" or "normal" mutation rate is probably chemically induced by the cell's own metabolites, with a small part of it being due to chance perturbations within the gene itself.

As to the precise chemical nature of the genic material, very little has been learned from the study of mutation other than the fact that the absorption spectrum of nucleic acid and the action spectrum of ultraviolet light in causing mutation show a high degree of correspondence, according to some workers. This fact taken together with the observations that nucleic acids contribute a large part of the weight of chromosomes, and that the transforming principle of bacteria is apparently desoxyribonucleic acid, would indicate that nucleic acids form an important part of the structure of genes. The importance of other compounds such as protein in this connection should not, however, be underestimated.

CHAPTER 4

Inherited Chemical Differences

Structural and functional differences in plants and animals are invariably associated with chemical differences. Some of these chemical differences may be of a simple type, involving qualitative or quantitative changes in compounds which may be isolated and precisely identified, or they may be at a more complex level, involving proteins or even spatial organization of chemical compounds in cells and parts of the organism. In any event, whether the chemical differences are easily analyzed or not, the generalization stated above seems always to hold, and investigations into the nature of gene activity are invariably predicated upon it as the basic premise. Therefore, we shall begin our consideration of gene activity by inquiring into the inheritance of chemical differences.

An inquiry of this nature may involve at least two approaches: (1) the analysis of differences in chemical reactions, a dynamic approach; or (2) an analysis of the end products of these reactions, a more static approach. The two are, of course, related, since the product is the result of a reaction, but, in order to study the reaction properly, one must know the reactants as well as the end products, and this is not always possible even with the present advanced development of biochemistry. This chapter is therefore chiefly concerned with the inheritance of differences in chemical constitution. More specifically, it deals with the inheritance of those compounds for which the chemist and biologist have developed tests to determine qualitative and quantitative differences.

The main problems that confront the geneticist who desires to investigate inherited chemical differences are chemical rather than genetical. Therefore it is usually in investigations where there has been close collaboration between the chemist and the biologist that the soundest results have been obtained. There are many examples of

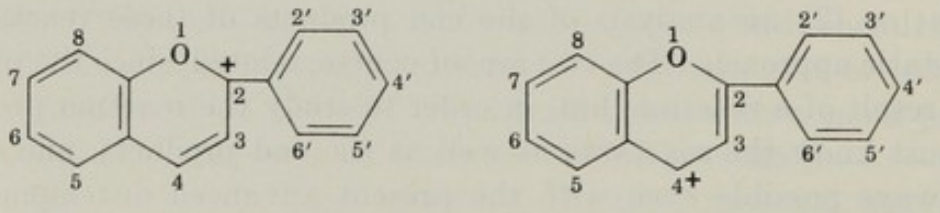
chemical differences reported in the literature in which the genetic side has been worked out thoroughly but in which the chemical aspect is poorly developed, such as the studies in animal pigments, particularly the melanin pigments. A discussion of inheritance in examples such as these will be deferred to later chapters. First an attempt will be made to develop a background by referring to the inheritance of compounds which are chemically well enough understood for tentative conclusions to be drawn as to what is actually being inherited.

1. Anthocyans and Anthoxanthins in Plants

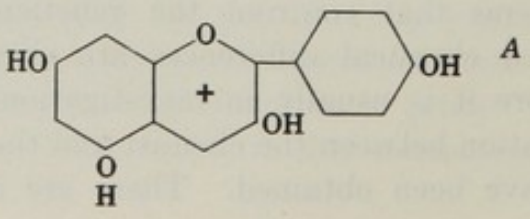
The anthocyans and anthoxanthins are water-soluble pigments found in solution in the cell sap of plants. They are responsible for most of the flower and fruit colors and in addition certain of the leaf colors occurring in the higher plants. Their chemistry is fairly well understood, and the ease with which they can be extracted in relatively pure state from plant material and identified with simple color tests has made them favorite compounds to study in connection with the inheritance of chemical differences in plants.

Chemistry

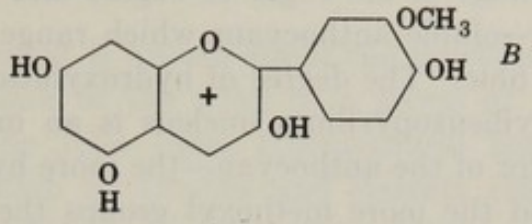
The anthocyans (or anthocyanins) are glycosides of polyhydroxy-2-phenylbenzopyrilium salts. The two resonating allylic structures illustrated have been shown to account for the ionic properties of the 2-phenylbenzopyrilium nucleus (560*a*). For convenience ionic structures will be indicated below with a + in the 1, 2, 3, 4 ring.



Hydroxylation of this nucleus gives a variety of compounds known as anthocyanidins, such as, for example, pelargonidin, or 3,5,7,4′-tetrahydroxy-2-phenylbenzopyrilium (*A*). Methylation or methoxylation



A



at the 3′ or both the 3′ and 5′ positions is responsible for the formation of several other types of anthocyanidins, such as peonidin (*B*), which is the anthocyanidin cyanidin (see Table 7) methylated at the 3′ position.

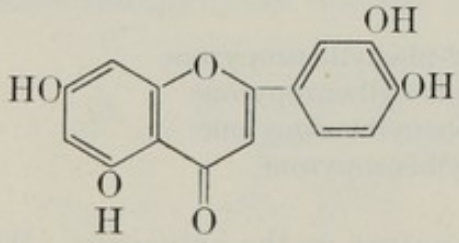
Table 7. Common Anthocyans and Anthoxanthins of Natural Occurrence

Anthocyanidins	
Pelargonidin	3,5,7,4′-Tetrahydroxy-2-phenylbenzopyrilium
Cyanidin	3,5,7,3′,4′-Pentahydroxy-2-phenylbenzopyrilium
Delphinidin	3,5,7,3′,4′,5′-Hexahydroxy-2-phenylbenzopyrilium
Peonidin	3,5,7,4′-Tetrahydroxy-3′-methoxy-2-phenylbenzopyrilium
Malvidin	3,5,7,4′-Tetrahydroxy-3′,5′-dimethoxy-2-phenylbenzopyrilium
Anthocyans	
Pelargonin	Pelargonidin-3,5-diglucoside
Cyanin	Cyanidin-3,5-diglucoside
Callistrephin	Pelargonidin-3-monoglucoside
Chrysanthemin	Cyanidin-3-monoglucoside
Primulin	Malvidin-3-monogalactoside
Peonin	Peonidin-3,5-diglycoside
Anthoxanthins	
Quercitin	3,5,7,3′,4′-Pentahydroxy-2-phenylbenzopyrone
Luteolin	5,7,3′,4′-Tetrahydroxy-2-phenylbenzopyrone
Kaempferol	3,5,7,4′-Tetrahydroxy-2-phenylbenzopyrone
Apeginin	5,7,4′-Trihydroxy-2-phenylbenzopyrone

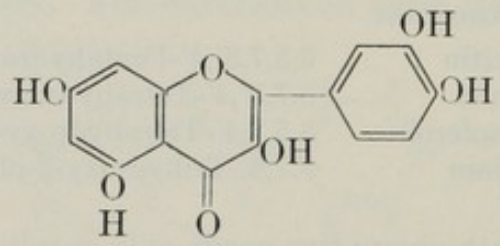
Anthocyanidins occur only rarely in nature as the aglycones. When free of the sugar, they are insoluble in water. Usually they are associated with the hexose sugars, glucose and galactose, or with disaccharides, such as gentiobiose. The sugar molecule or molecules are bound to the anthocyanidin in glycosidic linkage. The methyl pentose sugar, rhamnose, is occasionally found involved, as are compounds formed by acylation with organic acids, such as *p*-hydroxybenzoic acid, malonic acid, *p*-coumaric acid, and *p*-hydroxycinnamic acid. Either one or two sugar molecules may be attached to the anthocyanidin. One of the sugars is nearly always at the 3 position; the other, if present will be at the 5 position.

The chemical linkage of the sugar or sugars and the anthocyanidin produces the water-soluble anthocyans which range in color from red through violet and blue. The degree of hydroxylation and methoxylation of the 2-phenylbenzopyrilium nucleus is an important factor in determining the color of the anthocyan—the more hydroxyl groups the bluer the color, and the more methoxyl groups the redder the color. The addition of another sugar molecule to a monoglucoside anthocyan increases the blueness. The *p*H of the cell sap is also a contributing factor, for the anthocyanidins are color indicators. For example, cyanin is red at *p*H 3.0, violet at *p*H 8.5, and blue at *p*H 11. In addition to these factors, the final color of flower petals containing anthocyans is modified by the presence of anthoxanthins or occasionally tannins. This is not caused alone by the blending of the anthocyan with the anthoxanthin, but by a weak chemical linkage between the molecules of the two types of pigments which results in the phenomenon of copigmentation. In general, copigmentation results in a bluer color than would be the case if the anthocyan were alone.

The anthoxanthins apparently occur in plants as either the aglycones or as glycosides. Their structures are similar to those of the anthocyans; but they are oxidized in position 4. Hydroxyl groups at positions 5 and 7 seem to be the rule just as in the anthocyans, but two groups of anthoxanthins have been defined differing only in the occurrence of a hydroxyl group at position 3. These are the flavonols and the flavones.



Luteolin (flavone)



Quercitin (flavonol)

The anthoxanthins vary from pale ivory to intense yellow. In general, an increase in the number of hydroxyl groups on the 2-phenylbenzopyrone nucleus increases the intensity of the yellow color, but the effect of additional sugar molecules does not seem to be known. There is little or no effect of *p*H on the color produced by the anthoxanthins.

Inheritance

A large part of what is known about the inheritance of flower color pigments is the result of the work of a group of English geneticists and chemists at the John Innes Horticultural Institution. Starting with the investigation by Onslow and Bassett (464) in 1913 of the flavone pigments of the snapdragon, *Antirrhinum majus*, these workers have in the ensuing years established the chemical basis of inherited flower color differences in a large number of cultivated plants. As the results of their extensive investigations are adequately reviewed by Lawrence and Price (350) and Lawrence (349), we shall confine ourselves here to a discussion of only a few examples.

Drastic modification of flower color may be produced by a change in a single gene. For example, whether the snapdragon has white flowers or yellow depends upon the particular combination of a pair of alleles, *Y* and *y*, yellow-flowered plants being of the genotype *YY* or *Yy*. The yellow color in this case results from a mixture of two anthoxanthins, luteolin and apeginin (464, 465), which are absent or at least undetectable in the recessive, white *yy* plants. Other examples in which the presence of a pigment as opposed to its absence is determined by a single dominant gene are given in Table 8. It will be noted that in

Table 8. Some Examples of Simple Inheritance of Flower Color in Which the Presence of a Pigment Is Determined by a Dominant Gene

Pigments Present in Dominant	Recessive Phenotype	Gene Designation	Plant	Reference
Luteolin and apeginin (yellow)	White	*Y*	Snapdragon, *Antirrhinum majus*	464
Malvidin (magenta)	Ivory or yellow	*B*	Primrose, *Primula acaulis*	84
Cyanidin or pelargonidin	Yellow	*C, R*	Wallflower, *Cheiranthus cheiri*	554
Cyanidin, pelargonidin, or delphinidin	White	*C, R*	Sweet pea, *Lathyrus odoratus*	554
Cyanidin or pelargonidin	Yellow or white	*Y, A*	Carnation, *Dianthus caryophyllus*	193

three of these examples, the primrose, wallflower, and carnation, the recessive flowers may be colored ivory or yellow. This is because the background anthoxanthin pigment is unaffected by the gene substitutions which in these three cases cause detectable changes only in anthocyan. The particular type of anthocyan which is present is determined by other genes.

In the wallflower, sweet pea, and carnation there are two genes involved, which must be present together in the dominant condition for pigment formation of the type indicated. Thus sweet pea plants of the genotype *CCRR*, *CcRr*, *CcRR*, *CCRr* all have flowers containing anthocyans, while *ccrr*, *Ccrr*, *CCrr*, *ccRR*, and *ccRr* plants have none. *C* is therefore said to be *complementary* to *R*, and vice versa, in the sweet pea and wallflower. *Y* and *A* have the same relationship in the carnation.

The inheritance of qualitative differences among the anthocyans has been extensively studied in a number of species. Three significant types of changes in the anthocyan molecule have been found to be inherited in a simple Mendelian fashion: (1) hydroxylation, (2) methoxylation, and (3) number of sugar molecules attached to the anthocyanidin nucleus. The Cape primrose (*Streptocarpus* sp.) hybrids, investigated by Lawrence, Scott-Moncrieff, and Sturgess (352), show in their inheritance all three of these differences, with three genes, *O*, *R*, and *D*, involved. Figure 22 illustrates the effect of substituting the dominant and recessive alleles *R* and *r*, *O* and *o*, and *D* and *d* in flower pigments in *Streptocarpus* hybrids derived from a cross between *S. Rexii* and *S. Dunii*, which are two pure-breeding wild strains with blue and red flowers respectively. Since the genes *R*, *D*, and *O* appear to be completely dominant to their respective recessive alleles, the genotype designations in the figure are shortened to the minimum necessary to describe the genotypic state. Thus *rod* is equivalent to *rroodd*, and *RoD* is equivalent to *RrooDd*, *RRooDD*, *RrooDD*, or *RRooDd*, and so on for the others. This type of shorthand is a convenience which will be used throughout this book wherever complete dominance appears to hold and a number of genes are involved.

When *r* is substituted for by *R*, it will be noted that a higher degree of oxidation is obtained both in those plants with a mixture of mono- and diglycosides (*rod*) and those with diglycosides only (*roD*). The oxidation may result simply in hydroxylation, in which case cyanidin derivatives are produced, or in methoxylation, with the production of peonidin derivatives. Further oxidation at both the 3′ and 5′ caused by the substitution of *O* for *o* results in methoxylation only, with the formation of malvidin derivatives. The state of *R* in *O* plants is unimportant, since both *OR* and *Or* plants are phenotypically identical with respect to the degree of oxidation. *O*, therefore, "masks" the effect of *R*, or is *epistatic* to *R*. The term *epistasis* is used frequently to describe the "dominance" of one gene over a non-allelic gene, as is exhibited here.

The effect of gene *D* is to cause the appearance of almost pure 3,5-diglycosides in place of a mixture of mono- and diglycosides. It does this whatever the states of the other two genes *R* and *O*.

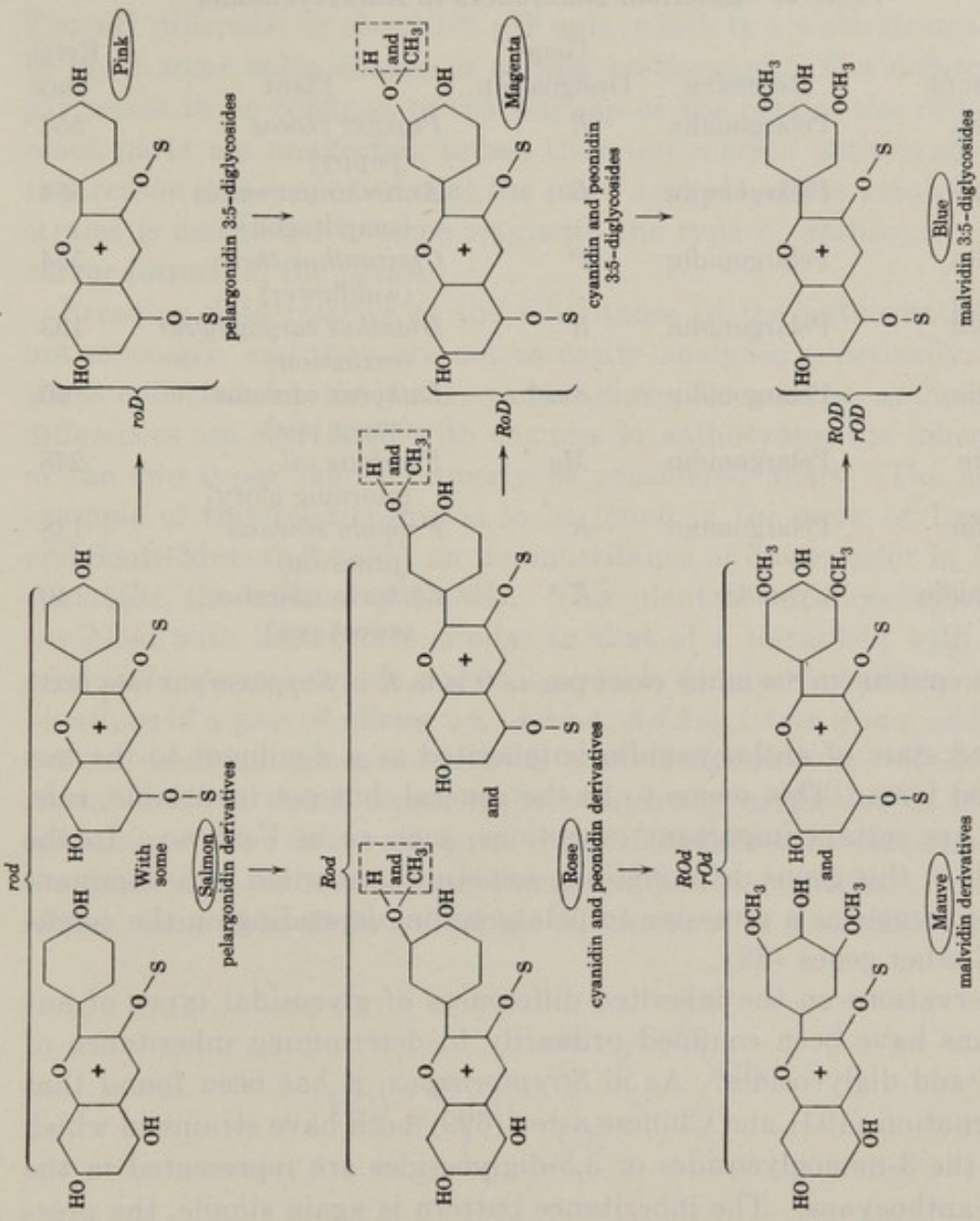


Fig. 22. The effect of gene substitutions in *Streptocarpus* sp. on the degree of oxidation and the number of sugars in the flower anthocyans. (S = sugar.) After Lawrence, Scott-Moncrieff, and Sturgess (352).

To summarize, it is clear that the action of *R* and *O* is to determine the degree of oxidation of the anthocyanidin. *R* results in oxidation, either hydroxylation or methoxylation, at the 3′ position, and *O* at both the 3′ and 4′ positions. *D* has nothing to do with the state of oxidation but determines an increase in the amount of 3,5-diglycosides.

The inheritance of qualitative differences in anthocyanidins as described for *Streptocarpus* is essentially similar to that found in many

other plants. A few of the more significant examples are listed in Table 9. Here it will be noted that, as in *Streptocarpus,* the more

Table 9. Inherited Differences in Anthocyanidins

Dominant	Recessive	Gene Designation	Plant	Reference
Cyanidin	Pelargonidin	*E*	*Papaver rhoeas* (poppy)	554
Cyanidin	Pelargonidin	*B*	*Antirrhinum majus* (snapdragon)	554
Cyanidin	Pelargonidin	*P*	*Cheiranthus cheiri* (wallflower)	554
Cyanidin	Pelargonidin	*R*	*Dianthus caryophyllus* (carnation)	193
Cyanidin	Pelargonidin	*Sm* *	*Lathyrus odoratus* (sweet pea)	40
Peonidin	Pelargonidin	*Mg*	*Pharbitis nil* (morning glory)	248
Malvidin	Pelargonidin	*K*	*Primula sinensis* (primrose)	138
Delphinidin	Cyanidin	*E* *	*Lathyrus odoratus* (sweet pea)	40

* *E* is epistatic to *Sm* in the sweet pea as *O* is to *R* in *Streptocarpus* (see text).

oxidized state of anthocyanidin is inherited as a dominant to the less oxidized form. This seems to be the general, but not invariable, rule. There are certain important exceptions, such as in *Verbena.* In the hybrids of this genus delphinidin is sometimes inherited as a dominant and sometimes as a recessive to pelargonidin, depending on the condition of other genes (38).

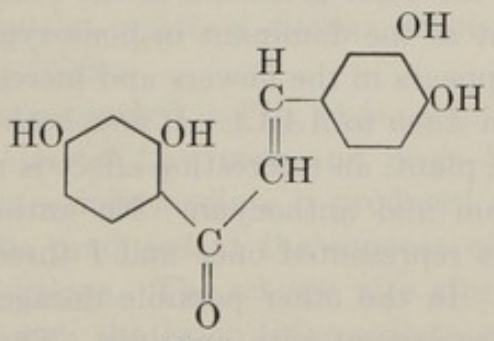
Observations on the inherited differences of glycosidal types of anthocyans have been confined primarily to determining inheritance of mono- and diglycosides. As in *Streptocarpus,* it has been found that the carnation (193) and Chinese aster (699) both have strains in which either the 3-monoglycosides or 3,5-diglycosides are represented in the flower anthocyans. The inheritance pattern is again simple, the presence of one or the other being due to a single gene with the 3,5-diglycosides dominant to the 3-monoglycosides. *Verbena* (38) is an exception, as it is in inheritance of degree of oxidation, for in some strains the diglycosides are dominant and in others recessive to the monoglycosides.

As mentioned previously, the *p*H of the medium in which the anthocyan is in solution may have considerable effect on the color. In many of the plant species which have been investigated, *p*H differences in

the flower petal cell sap have been found among the color varieties. In at least three of these species, *Primula sinensis, Papaver rhoeas* (554), and *Primula acaulis* (84), *p*H differences are inherited in a simple Mendelian fashion, with the lower *p*H dominant over the higher. The *p*H difference is about 0.5 *p*H unit, which is a wide enough range to cause some color change in certain anthocyans. The difference in *p*H seems to be confined to the cell sap of the petals; the rest of the plant parts are unaffected, unless they too contain anthocyans. For this reason it is suspected that the more acid *p*H of the petals in some strains is associated in some way with the type of anthocyan or precursor formed in the petals.

Breeding data relative to the inheritance of the anthoxanthins are not extensive, and they are not as easily analyzed genetically as are those collected for the anthocyans. Since, in general, anthoxanthin differences are correlated with changes in anthocyans, the inheritance of the two types cannot properly be considered apart. The clearest example of this relationship is to be found in the work of Lawrence and Scott-Moncrieff (351) on the inheritance of flower color in *Dahlia variabilis,* the cultivated *Dahlia.* This plant is an alloöctoploid (an octoploid with inheritance similar to that of a tetraploid with tetrasomic inheritance), which makes it possible to have five different combinations of a pair of alleles, i.e., *AAAA*, *AAAa*, *AAaa*, *Aaaa*, and *aaaa*.

Four dominant genes are known to affect flower color. Each has a recessive allele which seems to be completely ineffective in pigment production. The dominant genes *A* and *B* are directly concerned with the production of anthocyans containing either cyanidin or pelargonidin. Gene *Y* is accompanied by the presence of a chalkone, butein,



Butein

discovered in *Dahlia* by Price (496). The fourth gene, *I*, is related to the production of a flavone, apeginin.

Plants which are homozygous recessive for *a*, *b*, *y*, and *i*, i.e., *aaaa*, *bbbb*, *yyyy*, and *iiii*, are devoid of flower pigment, but the presence of

dominant alleles of any one or more of the four result in some pigment. However, the production of the three pigments together in one flower because of a dominant *A* or *B*, *Y* and *I* is not a simple matter (see Fig. 23). The amount of one pigment produced may be very much dependent upon the production of another. For example, in plants homozygous recessive for *y*, *a*, and *b*, but with dominant *I* present, apeginin appears in the flowers in an amount dependent upon the number of *I* alleles present. *Iiii* plants have a small amount, and the

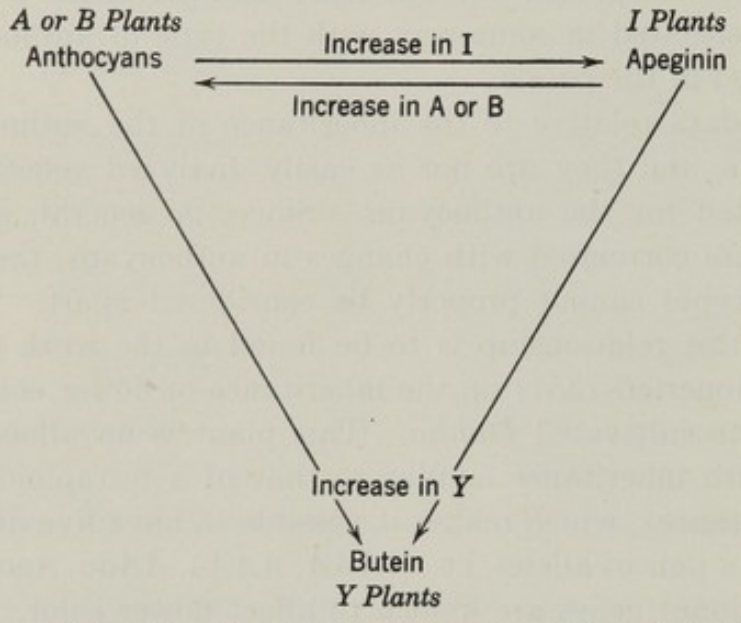


Fig. 23. The relationship of anthocyans, butein, and apiginin in *Dahlia variabilis* as determined by the genes *A*, *B*, *Y*, and *I*.

genotypes *IIii*, *IIIi*, *IIII* have equivalent and the maximum attainable amount. *I* is therefore completely dominant to *i* when it is present twice provided that the other genes are in the recessive state.

When *A* is present as the dominant in homozygous recessive *y*, *i*, *b* plants, anthocyan appears in the flowers and increases with increase in the dosage of *A* from *Aaaa* to *AAAA*. If now both *A* and *I* are present together in the same plant, an interesting effect is noted in the production of both apeginin and anthocyan. No anthocyan is present in plants in which *A* is represented once and *I* three or four times, but apeginin *is* present. In the other possible dosage combinations of *A* and *I*, anthocyans are present with apeginin. The relative concentrations of the two pigments are dependent upon the relative dosages of *A* and *I*. The introduction of *I* in increasing dosages into *A* plants causes a reduction in anthocyan correlated with an increase in apeginin, and, on the other hand, an increase in *A* is accompanied by an increase in anthocyan and a concomitant decrease in apeginin. Thus there exists an inverse correlation between anthocyan and apeginin produc-

tion which has been interpreted as meaning that the two types of pigments are competing in metabolism for a common, limited precursor substance. Further discussion is given this interpretation in Chapter 10. For the present it is sufficient to point out that a relationship in the inheritance of the two pigments is proved in *Dahlia variabilis.*

The production of butein in plants recessive for *a* and *b* shows a pattern similar to the one described above. Only a trace of apeginin but considerable butein is found in *Yyyy IIii* plants, whereas in *YYyy IIii* individuals apeginin seems to be completely replaced by butein. Furthermore, an examination of the effects of the possible dosage combinations between *Y* and *B*, and *Y* and *A*, reveals that butein is produced at the expense of anthocyan. Thus it is apparent that all three pigments, anthocyans, anthoxanthins, and chalkones, are related in their inheritance, and perhaps are therefore associated in biogenesis.

In order to correlate the genetic and chemical observations on flower pigments, Robinson (518) has proposed the existence of a colorless intermediate or leuco form which he hypothesizes is the precursor to all anthocyanidins, anthoxanthins, and related pigments. This compound and examples of its presumed derivatives are illustrated in Fig. 24. The presence of leuco compounds, either as postulated in Fig. 24 or similar in structure to it, in the petals of flowering plants, is well established (519). They are easily convertible to colored pigments by treatment with mineral acids. Furthermore, Stephens (599) has shown convincingly that there is a conversion of quercitin in Asiatic cotton (*Gossypium,* sp.) petals through an intermediate form, which is colorless, to cyanidin during the morphogenesis of the flowers. A corresponding reduction of quercitin to cyanidin may be carried out *in vitro* with the intermediate formation of a leuco compound with absorption in the ultraviolet which is similar or identical to the compound formed *in vivo* (600).

A scheme such as is presented in Fig. 24 is useful in interpreting the results found in *Dahlia* by Lawrence and Scott-Moncrieff, for it is evident that if the leuco intermediate is produced in limited amounts some pigments will be produced at the expense of others, depending on the metabolic conditions. The scheme also allows for interconversion of pigments through the leuco intermediate such as is found in cotton and indicated in other species. The manner of inheritance of the different types of anthocyanidins indicates that they may be derived from a common intermediate form such as cyanidin. Cyanidin is assumed to be the precursor of pelargonidin, delphinidin, and the various methylated derivatives, because of the fact that it is the most widely distributed anthocyanidin in nature (39). Furthermore, the

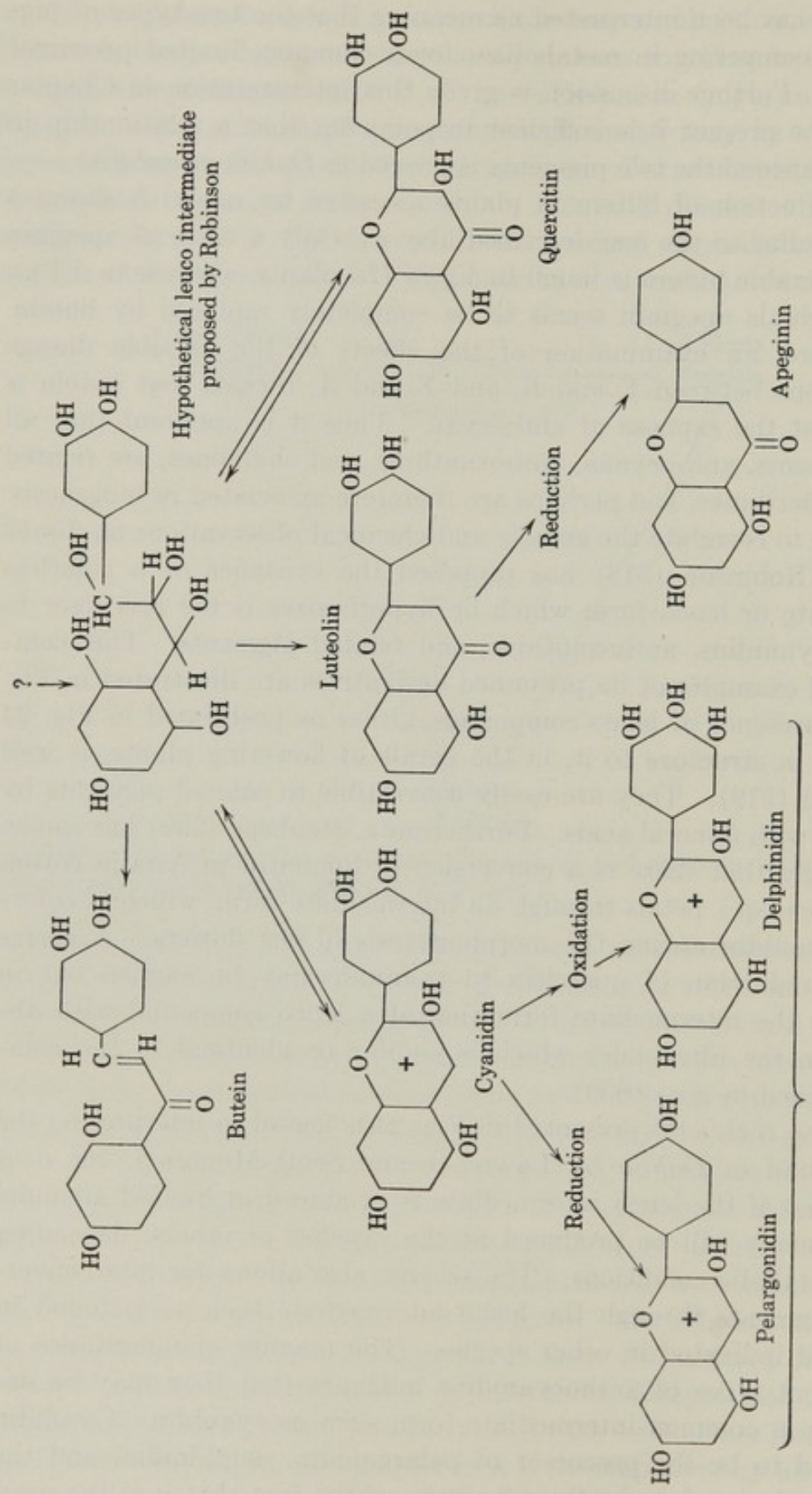


Fig. 24. Biosynthetic relationships postulated for anthocyanidins and anthoxanthins.

fact that it is found almost exclusively in the more primitive flowering plants indicates that it plays a primordial role in the origin of the other anthocyanidins.

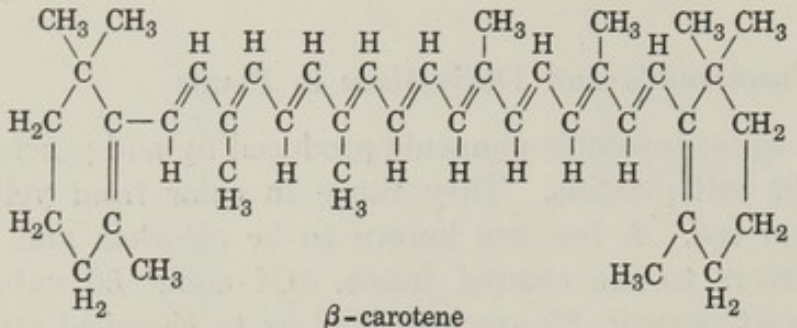
2. Carotenoids and Derivatives in Plants

Carotenoids are water-insoluble pigments produced by and generally retained within the cell plastids. They range in color from yellow through orange and red. A few are known to be colorless and are presumably precursors to the colored forms. Of about 80 natural carotenoids known at present, 35 are identified as to chemical structure (321). The basic carotenoid structure is a carbon chain having alternate single and double bonds (Fig. 25). The differences among the various carotenoids are those of stereoisomerism including *cis-trans* isomerism, the presence or absence of alicyclic rings at the ends of the chains, the number and arrangement of the double bonds, and the degree of oxidation. Carotenoids are generally divided into two groups: the carotenes, which are simple polyene hydrocarbons; and xanthophylls, the oxidized forms of the carotenes. In the green parts of plants both the carotenoid and the chlorophyll derivatives are present together in the chloroplasts, but the green of the chlorophyll masks the red or yellow of the carotenoids. The carotenoid colors are, however, readily recognized in the chlorophyll-free structures, such as the fruits of the tomato and the roots of the carrot, and in mutant forms in which the chlorophyll is absent but the carotenoids present.

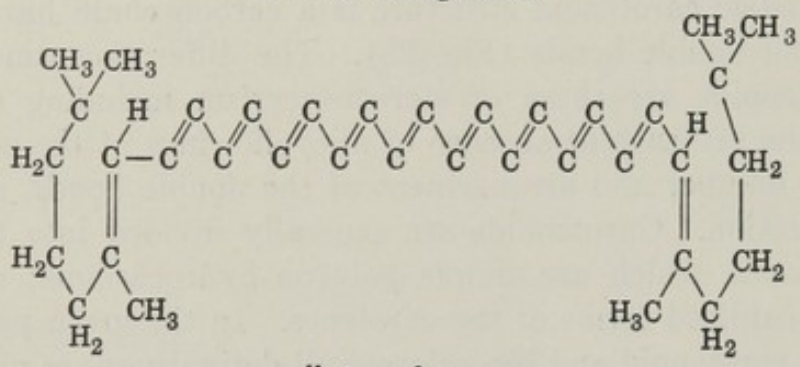
The pigmentation of the tomato fruit is produced by carotenes. *All-trans* lycopene, *cis* isomers of lycopene, and β-carotene seem to be the predominant types (Fig. 25). The familiar red tomato contains primarily *all-trans* lycopene and β-carotene, but there are other color varieties with qualitative and quantitative differences. Genetic analysis reveals that at least three genes are involved in the inheritance of these differences and that fruit color is related to two of these, *R* and *T*, as follows (315, 396, 397):

RRTT, *RrTt*, *RrTT*, *RRTt*	Red
RRtt, *Rrtt*	Orange (tangerine variety)
rrTT, *rrTt*	Yellow
rrtt	Intermediate between yellow and orange

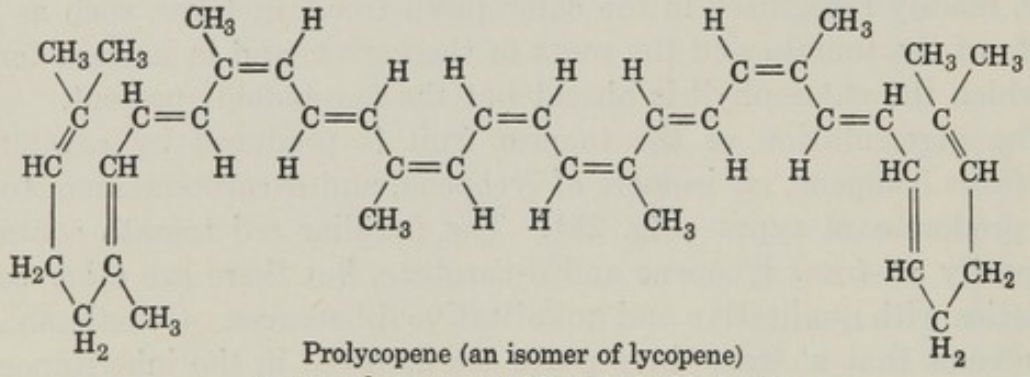
Table 10 presents some data of Mackinney and Jenkins (397) which show the quantitative and qualitative differences between the four



β-carotene
all-trans configuration



all-trans lycopene



Prolycopene (an isomer of lycopene)
presumed structure with 5 *cis* double bonds

Fig. 25. Structures of the principal types of carotenoids known to occur in the fruit of the tomato. The structure of *all-trans* lycopene is identical to β-carotene except for the absence of the rings at the ends of the chain. The hydrogens and methyl groups have been omitted from the middle part of the lycopene chain to emphasize the difference between it and the *cis* configuration of prolycopene.

phenotypes. It will be noted that yellow (*rT*) fruits are characterized by a very low total carotene content while the red (*RT*) and orange (*Rt*) contain the most carotene. The double recessive contains intermediate amounts.

Table 10.* The Carotene Content of Four Strains of the Tomato, *Lycopersicon esculentum*

	Micrograms of Carotenoid per Gram of Fruit			
Carotene Type	*RT* Red	*Rt* Orange	*rT* Yellow	*rt* Intermediate
all-trans Lycopene	70–130	—	0–0.5	—
cis Isomers of lycopene	—	40–55 †	—	10–15 †
β-Carotene (*all-trans*)	5–10	3–12	1–3	0.5–1.0
poly-cis-Carotene	—	8–15	—	—
all-trans-ζ-Carotene	0–0.1	20–15	—	0.01
Phytofluene ‡	3–5	4–7	ca. 0.1	0.7–1.0
Total carotenes	80–150	75–150	3–7	15–20

* From Mackinney and Jenkins (397). See also Tomes et al. (656).

† Half or more prolycopene, the rest mono-*cis* isomers.

‡ A colorless carotene presumed to be a possible precursor to the colored compounds.

Thus, total carotenes considered, the following quantitative order is found: $rT < rt < Rt = RT$, showing that the major effect of substituting R for r is to increase the total amount of carotene. If now the qualitative differences are analyzed, it is seen that *cis* isomers occur in detectable amounts only in those plants in which t is homozygous, namely those bearing orange- and intermediate-colored fruit. In these it is also evident that *all-trans lycopene* is absent, although β-carotene and ζ-carotene are present to represent the *trans* isomers. Because of the association of *cis* isomers with *tt*, Zechmeister and Went (722) have suggested that the *T* gene specifically determines the stereoisomeric configuration of the carotene molecule. This is almost certainly an oversimplification of the true situation, but it does serve to point out the possible types of chemical changes accompanying single gene changes.

A third gene affecting fruit color in the tomato, *B*, and its partially recessive allele *b*, have been described by Lincoln and Porter (377). These workers employed tomato strains of the residual genotype, *RRTT*, to show that substitutions at the *B* locus had the following effects on the carotenes:

RRTTBB, 93% of total carotene β-carotene
RRTTBb, 61% of total carotene β-carotene
RRTTbb, 10% of total carotene β-carotene

The total carotene content is the same for all three genotypes, *RTBB*, *RTBb*, and *RTbb*, but the lycopene content is reduced as the β-carotene increases. This suggests either that β-carotene and lycopene are formed from the same limited precursor, or that β-carotene is naturally synthesized from lycopene, a process which would involve closing the rings (see Fig. 25).

An even more definite quantitative effect of gene substitution on the amount of a carotenoid produced in a plant is to be found in the inheritance of vitamin A activity of corn endosperm. Since endosperm is a triploid tissue, four different combinations of *Y* and its allele *y* which affect vitamin A production are possible (403).

Genotype of Endosperm	Vitamin A Activity
yyy	0.05
Yyy	2.25
YYy	5.00
YYY	7.50

A single dose of the gene *Y* determines the presence of about 2.50 units of vitamin A activity, and the effect of the gene is additive, while *y* has little or no effect.

3. Compounds Present in Corn Endosperm

The chemical content of corn endosperm has received the attention of agronomists for many years, since it is in this tissue that the primary nutritive value of the corn kernel lies. Corn endosperm (see Fig. 14) has several advantages for chemical and genetical analysis. It is easily separated from the embryo tissue, thus enabling the chemist to work with relatively uniform tissue contaminated only by the presence of a thin outer layer of pericarp, a maternal sporophyte tissue. It possesses, as does the endosperm of many other plants, a triploid number of chromosomes, thus making it possible for the geneticist to vary the dosage of genes affecting endosperm over a wider range than is possible in the diploid tissues and so determine more precisely the additive effects of these genes on the phenotype in a manner similar to that described for *Dahlia* (p. 81).

The carbohydrate, protein, fat, and vitamin content of corn endosperm can be changed greatly by means of proper breeding techniques. For example, the carbohydrate content is dependent to a large extent upon the action of genes at two independent loci, *dull* with alleles *Du*,

du, and *sugary-1* with alleles Su_1, su_1, and su_1^{am} (402). Cameron (87) has made detailed chemical analyses of the carbohydrate in corn endosperm with these genes present in different dosages, and found that su_1^{am} (*sugary amylaceous*) and *Du* determine the presence of a high content of soluble carbohydrates (both soluble polysaccharides and simple sugars). Their effects are additive; and substitution by the respective recessive alleles, su_1du, results in a decrease in starch and an increase in soluble carbohydrate. Besides changing the carbohydrate content these genes also alter the physical appearance of the kernels and the concentration of other chemical compounds. The kernels with large amounts of starch are opaque and smooth in appearance, while those with high sugar are translucent and wrinkled. Table 11 lists supplementary data on the nicotinic acid, thiamine,

Table 11.* Carbohydrate and Vitamin Differences in *Dull* (*du*) and *Sugary* (su_1 and su_1^{am}) Corn Endosperm

Genotype of Endosperm	Phenotypic Description	% Starch	Total † Sugars %	µg/gm of Dry Tissue: Nicotinic Acid	Thiamine	Biotin
$su_1^{am}su_1^{am}su_1^{am}DuDuDu$	Primarily starch, opaque and smooth	80.7	0.08	21.7	0.18	0.07
$su_1^{am}su_1su_1Dududu$	Primarily starch, opaque and smooth	58.6	0.42	29.4	0.37	0.09
$su_1^{am}su_1^{am}su_1^{am}dududu$	Sugary, translucent, and wrinkled	51.5	0.82	44.3	0.42	0.14
$su_1su_1su_1DuDuDu$	Sugary, translucent, and wrinkled	32.2	2.63	56.3	0.98	0.13
$su_1su_1su_1dududu$	Sugary, translucent, and wrinkled	19.2	2.59	56.7	0.88	0.15

* From Cameron (87) and Cameron and Teas (88).
† Sucrose and reducing sugars.

and biotin content of the various types of kernels as determined by Cameron and Teas (88). It will be noted that these vitamins, like the sugars, increase in the kernels with decreasing dosages of su_1^{am} and *Du*. It is highly probable that other compounds are also affected by the *dull* and *sugary* genes, but further chemical analysis will be necessary to establish this.

The correlation between the increase in soluble sugars and in vitamin content of the kernels has no direct chemical basis. Teas (643) has shown that the nicotinic acid-rich aleurone layer of the sugary kernels is thicker than in the starchy kernels. The concentration of aleurone nicotinic acid is the same in both genotypes, and hence the increase in nicotinic acid at least is the result of a morphological effect of the *sugary* and *dull* genes rather than a direct chemical one.

Other mutant genes affecting the appearance of the kernels and structure of the endosperm have been investigated for their effects on the three related compounds, tryptophan, nicotinic acid, and indoleacetic acid (645). Table 12 shows the mutant effect as compared to

Table 12.* Average Tryptophan, Nicotinic Acid, and Indoleacetic Acid Contents for Mutant and Normal Corn Endosperms. Average Ratios of Mutants/Normal †

	Tryptophan		Nicotinic Acid		Indoleacetic Acid	
Mutant	Per Gram	Per Kernel	Per Gram	Per Kernel	Per Gram	Per Kernel
su_1 (sugary-1)	1.33	1.00	2.33	1.70	1.55	1.13
bt_1 (brittle-1)	1.83	0.85	5.12	2.41	2.58	1.33
bt_2 (brittle-2)	2.89	2.06	2.03	1.43	2.25	1.56
sh_2 (shrunken-2)	2.66	1.27	3.48	1.65	2.11	1.06
mn (miniature)	2.43	0.54	1.65	0.36	0.64	0.15

* Teas and Newton (645).

† Mutant and normal kernels picked from same ears to reduce genetic variability at other gene loci.

the dominant normal for five of the strains investigated, the mutant to normal ratio being given per gram and per kernel, since the mutant kernels are frequently smaller than the normal. There are obvious differences between the normal kernels and the mutant kernels with respect to these three compounds. It is of interest here, as in the previous example given for carbohydrate content, to note that mutant genes affecting the morphological condition of the kernels also produce chemical changes within the kernels.

4. Proteins and Other Compounds with Antigenic Properties

In the previous sections we have dealt with the inheritance of relatively simple chemical compounds for which the chemist can determine qualitative and quantitative differences by means of well-established analytical techniques. Other compounds or complexes of compounds may also be determined but by methods quite different from the standard chemical methods of analysis. For example, the proteins and other complex molecules of an organism, although almost impossible to analyze by ordinary chemical methods without destroying their identity, can be differentiated from one another to a limited extent by immunological reactions and the use of electrophoresis. The applica-

tion of the electrophoretic method, which entails the measurement of the rate at which a molecule moves in an electric field, has provided some interesting and valuable data about inherited chemical differences, some of which are described on p. 159. Most of what is known about differences in protein at the biological level is a result of the application of the immunological technique to the identification of proteins. The study of the inheritance of immunological differences has developed into an important field of genetic investigation, largely owing to the efforts of Irwin and coworkers, who have termed this branch of genetics *immunogenetics.*

The method of identifying proteins by immunological means is based upon the capacity of organisms to produce antibodies in response to foreign bodies known as antigens. The antigen may be a protein (and nearly all proteins act as antigens), or it may be a polysaccharide, lipid, or even simpler compound attached to a protein molecule. The significant fact about antibody formation which makes immunology such an important tool in the identification of biologically important complex compounds is that the antibody produced in the presence of an antigen is specific to that antigen and will react with no other antigen. There are important exceptions to this generalization, based on the degree of relatedness of the inducing antigens, which will be discussed below. On the whole, however, the generalization is a useful one when the exceptions are borne in mind.

If it is desired to compare the proteins or other antigenic substances of two individuals of the same or different species with the object of finding whether they have any in common, one proceeds by enlisting the aid of an acceptable unrelated animal such as a rabbit and injecting it with a preparation of cells or blood serum from one of the organisms under study. Since the injected material contains the antigens, which to the rabbit will constitute "foreign bodies," it will react by producing antibodies to them. After this has happened (the rabbit is then said to be immune to the antigens), the rabbit serum is tested against the antigens from the second organism. If this organism has any antigens identical with those found in the first, the rabbit will have formed antibodies specific for them, so that a reaction will occur which will usually manifest itself as a precipitation, or an agglutination if the antigens are attached to cells such as the red blood corpuscles. If no discernible reaction occurs, it is then assumed that the two organisms have no antigens in common. The possibility that the unrelated intermediate organism may be incapable of forming antibodies to certain antigens must be taken into consideration, of course,

but experiments have shown that almost any material, provided that it is attached to a protein molecule and constitutes a "foreign body," will act as an antigen. This includes substances that are synthesized in the laboratory and which are not known to occur in nature.

It should be emphasized that antigenic differences do not always mean protein differences. Serum antigens are most probably proteins, but the cellular antigens are probably non-protein in nature, the protein portions acting as the bearers for the portions with the antigenic activity. It is not always possible to determine whether the specificity resides in a protein or non-protein or even in the combination compound, so that it can be said only that differences in immunological specificity are involved. This is more than screening ignorance with words, for actually the knowledge of antigens and antibodies has developed to an extent where it is possible to assert that immunological differences represent fundamental and important differences in the chemical structure of cells and tissues at the macromolecular level (658, 682), chemical differences of a complex type as yet only vaguely understood in terms of folding of long chains, types of surfaces, and so forth, but probably basic to differences exhibited at higher levels of organization.

The antigenic differences between a large number of different plants and animals ranging from the primitive single-celled forms on up to the most highly developed species have been investigated. The first generalization that emerged from this work was the discovery that many antigens are species specific. This was followed by the observation that there are also some similar antigens distributed among different species, but that the closer the relationship between the species, as determined by morphological similarities, the more antigens they hold in common. The first *intraspecific* differences in antigens in an animal were discovered by Landsteiner (341, 342) when he recognized the now well-known A, B, AB, and O blood groups in man. Individuals designated as A carry antigens *A*, those with antigen *B* as B, and those with both antigens as AB. A part of the human population carries neither antigen and is designated as O. These antigens are *cellular* antigens carried on the surface of the red blood corpuscles. Genetic analysis has made it certain that there is a series of at least three allelic genes involved in the inheritance of the antigens (688). The alleles I^A and I^B determine the presence of *A* and *B* antigens respectively, and the third allele I^O is incapable of producing either antigen. Both I^A and I^B are dominant to I^O, so that I^AI^O and I^BI^O individuals are phenotypically indistinguishable from I^AI^A and I^BI^B genotypes.

Persons heterozygous for I^A and I^B are of blood type AB so that it may be said that I^A and I^B are independent of one another in their action, neither one being dominant to the other. The blood type O is limited to those of the genotype I^OI^O. Various subgroups have been described, each being dependent upon the presence of an allele belonging to this series (688).

The early work on the inheritance of antigenic differences indicated that each definable antigen produced by an organism is inherited as a single gene character. The gene determining the character was in all cases found necessary to be present only once for the antigen to be formed, as in the blood type example given above. Furthermore it was found that the antigenic differences within a species could be classified as to whether they were inherited as allelic or non-allelic differences. For example, an organism producing 10 demonstrable antigens may, on genetic analysis, be found to inherit them through only 2 identifiable loci. Thus 6 of the antigens may be produced by a series of allelic genes, A^1, $A^2 \cdots A^6$, and the remaining 4 by an independently segregating group, $B^1 \cdots B^4$. This would mean that a considerable number of combinations are possible, but that at most only 2 of each group could be present in each individual of the species. It is now well established, however, that the inheritance pattern is more complicated than originally deduced, for it is apparent that a number of antigens are inherited in groups or sets, such as $A_1A_2A_3$ or $A_2A_3A_5$, etc., as a genetic unit with no indication of crossing over. Either the previous generalization that each antigen is determined by a single gene must be modified, or the assumption must be made that each antigen of the set is determined by a single gene closely linked to other genes determining antigens in the same set.

The inheritance of certain red blood cell antigenic differences in cattle (617), chickens (76), ducks (395), and man (499, 689) demonstrates the type of "linkage" described above. Antigenic inheritance in cattle may be considered as an illustration. In this species at least 40 distinct antigenic factors are associated with the red blood cells of the Holstein-Friesian and Guernsey breeds (467). Breeding results have shown clearly that, of 38 of the known factors, 21 are inherited together in the "B system" and 7 in the "C system" (617). The remaining 9 are excluded from the B and C systems and await further genetic analysis. Eighty different inherited combinations of the 21 B antigenic factors have been identified, and 22 combinations have been found in the C group.

Figure 26 summarizes some of the data obtained and provides further explanation. Here each *antigenic factor* or group of factors inherited as a genetic unit is designated as a *blood group* or *antigen,* following the terminology of Stormont, Owen, and Irwin (617), which defines the term antigen to mean any group of serological components or anti-

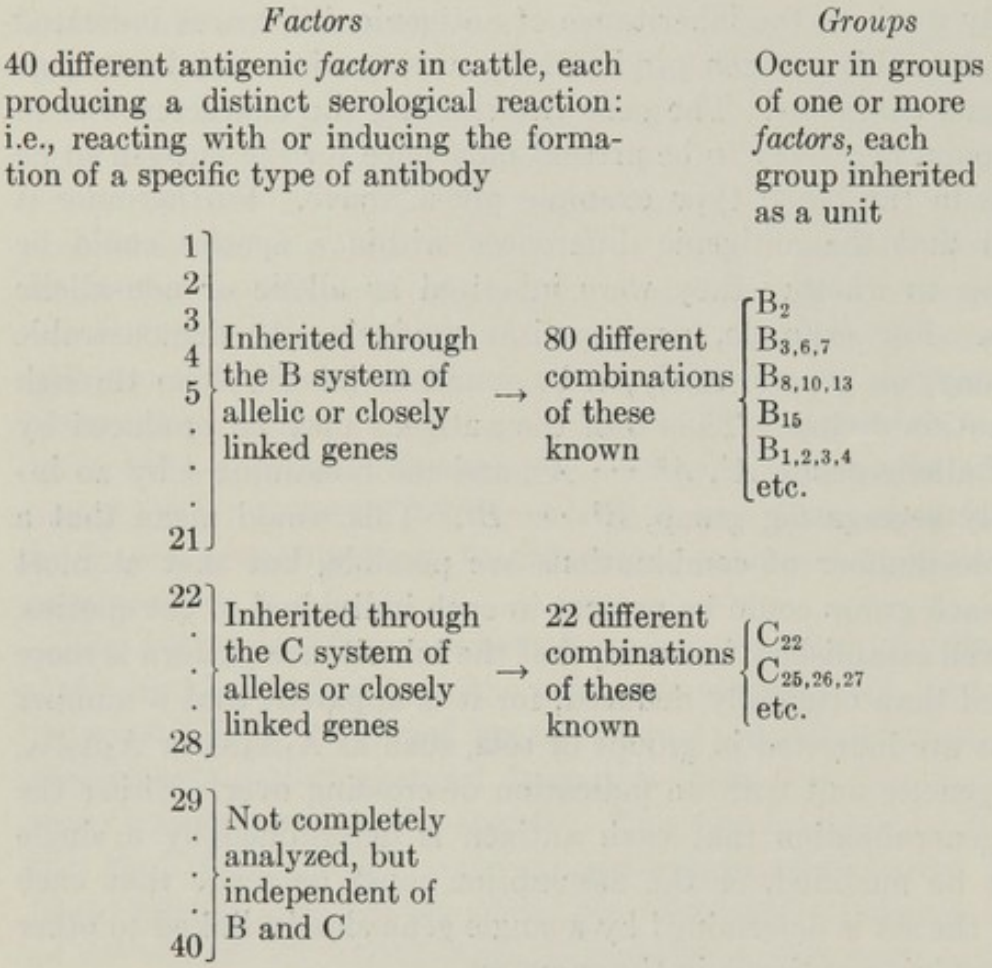


Fig. 26. The inheritance pattern for certain cellular antigens in cattle.

genic factors inherited as a unit. Thus a blood group or antigen is a complex of factors some of which may be common to other antigens. If each antigen with its single or multiple factors is considered as being determined by a single gene, there would then be two allelic series: B^2, $B^{3,6,7}$ $\cdots$ etc., with 80 alleles and $C^{22,26}$ $\cdots$ etc., with 22 alleles. Alternative genetic explanations are that non-allelic genes are involved, each determining a single antigenic factor, as discussed above, or that several loci, closely linked, and each with a number of different alleles, produce the antigenic patterns noted. The first alternative would require that the B system consist of 21 closely linked loci or "subgenes"

each with two alleles, one determining the presence and the other the absence of the antigen.

The present data for cattle and other forms, however, would indicate that the best explanation is that allelic series are involved, and that each allele of a series produces an antigen which is closely related in its serological properties to antigens produced by genes allelic to it. Each related antigen may be expected to stimulate the production of an antibody type which may cross-react in varying degrees with some or all of the related antigens. That cross reactions of this nature exist is abundantly proved by facts gathered from various sources. See Landsteiner (343). If cross reactions are involved in the serological differences demonstrated in cattle, then it need be assumed only that each allele of a given gene produces an antigen with serological specificities overlapping in varying degrees the antigens produced by the other alleles. This explanation satisfies the current data in cattle (616, 617) and may also prove to be a satisfactory explanation for similar cases in man, chickens, and ducks.

Although the principal work in immunogenetics has been done on the inheritance of cellular antigens located on the surface of red blood corpuscles, this does not mean that only differences in cellular antigens are inherited. The human erythrocyte antigens are also found in the saliva and in other body tissues, where they are inherited in the same way as described for the cellular antigens (688). Furthermore, tests made on the serum antigens of related dove species and their hybrids show that the species specific serum antigens present in the parents are found in the hybrids and are inherited in the same fashion as the cellular antigens (123).

As might be expected from the dosage effects of genes controlling pigment production discussed in the preceding sections, the presence twice of a gene determining a specific antigen results in more of that antigen than when the gene is present once in heterozygotes. Such quantitative differences between heterozygotes and homozygotes have been noted in most studies of human blood groups such as MNS (344, 536), Rh (399), and Duffy (500), and in the antigen studies on *Paramecium* (see p. 295). A competition for common substrate material is again the most likely explanation for this phenomenon as it is in the examples of plant pigments.

Another type of difference between heterozygotes and homozygotes is found in the inheritance of antigenic differences among doves (78) and ducks (394), in which hybrids formed by matings between species

with different antigens frequently produce "hybrid substances" which produce antisera specific for antigen found in the hybrids but not in either parent strain. Thus the dove, *Columba livea,* has an antigen C, while the related species *C. guinea* produces antigen C′. The two antigens are presumably produced by allelic genes. The hybrid produces an antigen CC′ which, however, is different from either C or C′, as demonstrated by tests with antisera. One possible interpretation of this phenomenon is that the alleles *C* and *C′* interact when together in the same genome to produce a single substance CC′ with different properties from either C or C′. It is but one example among many which can be interpreted in this way. Further consideration is given to allelic interaction in Chapter 9.

A question of fundamental importance, for which a clear answer is much to be desired, is the following. What is the relationship between inherited antigen differences and the more obvious chemical differences? Antigenic differences have not been directly related to specific differences in morphology and metabolism, although it is well known that different genotypic strains of any species almost invariably show differences in antigenic constitution. Attempts have been made to correlate differences in antigenic composition with gene differences causing changes in structure and chemical composition. The results have been promising, but inconclusive. For example, high egg production in chickens has been correlated with the presence of a specific antigenic complex, as shown by the fact that selection for this characteristic results in concomitant selection for the particular antigens (561). This may be a reflection of a direct relationship between characteristic and antigen. On the other hand, it could also mean selection of a whole complex of genes with different functions directed toward a common end (see Chapter 10).

Antigenic differences between wild-type and eye-color mutants have been demonstrated for *D. melanogaster* (184) and the Mediterranean flour moth, *Ephestia* (93), but it is impossible to make a decisive correlation between antigen and morphological characteristics from the data that have been presented. For one thing it is extremely difficult technically to produce a mutant strain differing from wild-type by a single gene difference. The oft-stated claim that one strain differs from another by a single gene always carries with it the usually unstated qualification that they differ in only one *known* gene. Other genes that have no morphological effect on the phenotype may differ as well, but produce antigenic differences unrelated to the visible effect of the mutant gene.

5. Summary of the Effect of Mutation and Dosage Changes on Chemical Constitution

It is established by the foregoing discussion that single gene changes can produce definite and specific chemical changes in constitution. What is not established is the nature of the metabolic changes resulting in chemical differences, nor, as emphasized in Chapter 3, is there much known about the nature of the mutations which precede and cause the

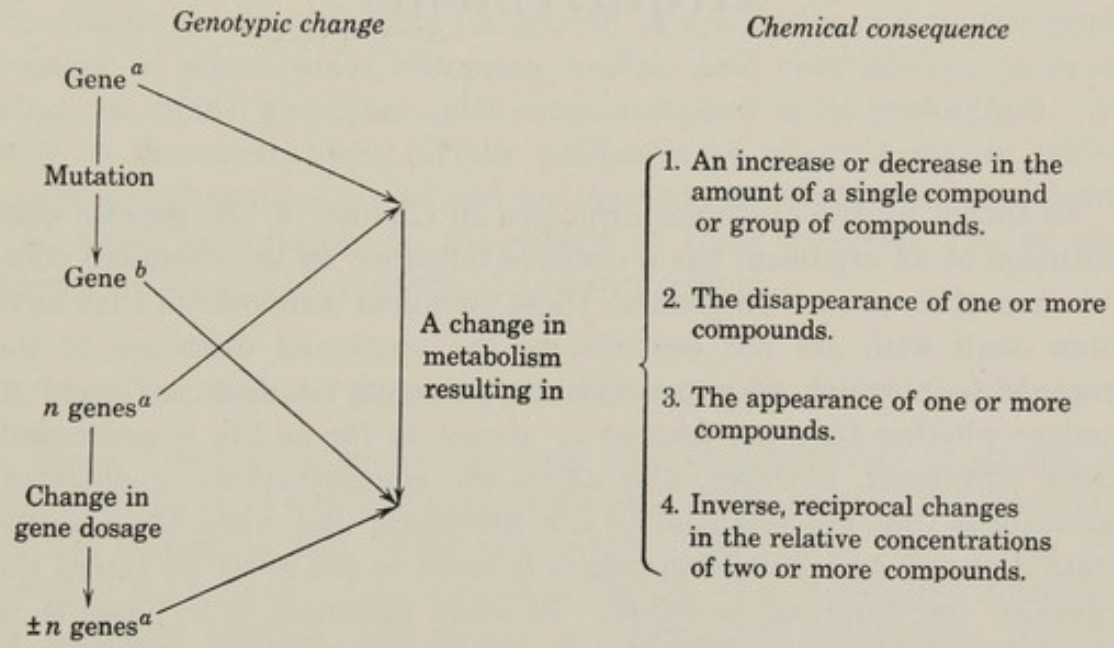


Fig. 27. Summary of the effect of gene mutation and changes in dosage on chemical constitution.

metabolic changes. It should be evident, however, that recognition of an inherited chemical difference and its nature is an important step toward ultimate understanding of the changed metabolism which effects that difference and, eventually, of the genic change which lies behind the whole chain of events culminating in the phenotype.

Figure 27 summarizes some of the observed chemical consequences of substituting one allelic gene for another or varying the dosage of a gene. The four types of chemical change indicated are not mutually exclusive for any one case of gene change, for the total change in chemical structure cannot be properly analyzed with the present chemical techniques. Furthermore, only the obvious changes are measured, and it is important to recognize that significant unidentified variations may accompany all the identifiable inherited differences described in this and subsequent chapters.

CHAPTER 5

Inheritance of Nutritional Requirements

As shown by the examples discussed in Chapter 4, the genetic constitution of an organism has a decisive influence on the chemical composition of its parts. In general, those chemical components that have been dealt with are not essential to the continued existence of the organisms in which they occur, and frequently it does not seem to matter whether they are present or absent so far as life is concerned. Gene mutations, however, also affect the concentrations of chemical substances that are essential to life processes, and when the concentration of any one such substance is reduced to too great an extent the effect of the mutation is lethal. In some instances it is possible to supply a missing essential compound from the external environment and the organism will continue to live and grow, sometimes in an apparently normal fashion. The study of such strains which have acquired nutritional requirements by mutation provides another valuable approach to the evaluation of the effects of genetic changes, although in principle it is no different than direct observations of changes in flower pigments. In practice this approach has been applied most successfully to studies of mutants of microorganisms. It originated with the investigations of Beadle and Tatum (31, 32), using the fungus *Neurospora* as the experimental organism, and it has since been used extensively with many other molds, yeasts, and bacteria.

As discussed here and much more extensively in Chapter 8, nutritional mutants have proved very valuable in studying pathways of biological synthesis of nutritionally important metabolites, since, in effect, different mutations appear to block different chemical steps in the many series of biochemical reactions that make up the patterns of metabolism. It should be recognized, however, that this method, like

others, has its own characteristic limitations. Mutations concerned with nutritionally important substances that will not pass through cell membranes are eliminated as lethals. It is also the usual practice to discard mutants that have obviously partial nutritional requirements, since these are inconvenient for many experimental purposes.

It has been advantageous to use organisms with simple growth requirements, since these have capacities for wider varieties of biosynthesis. *Neurospora,* for example, will grow on a medium containing inorganic salts, sugar (as a carbon and energy source), and the one B vitamin, biotin. During its growth it synthesizes all of the great variety of amino acids, vitamins, nucleic acid constituents, polysaccharides, lipids, and other substances contained in its protoplasm. As will be discussed, many of the pathways of biosynthesis by which essential metabolites are formed are very similar in different organisms, and much information on the details of these processes can be obtained by studies of mutants of microorganisms.

1. The Techniques of Inducing and Screening for Nutritional Mutants

Since *Neurospora crassa* has been used so extensively in studies of the inheritance of nutritional requirements (290), some of the essentials of the principles and techniques involved are given here. The life cycle of the fungus is diagrammed in Fig. 28. The vegetative mycelium is normally haploid, and it can be propagated indefinitely through transfers of fragments of the mycelium or the asexual spores to fresh medium. The mycelium forms two kinds of asexual spores, the macroconidia, which contain 4 or 5 nuclei on the average, and the uninucleate microconidia. Of the two mating types which are designated *A* and *a*, either will produce fruiting bodies or protoperithecia on an appropriate medium (685). After these have been produced, the sexual cycle is initiated by fertilization with conidia or mycelium of the mating type opposite to that of the protoperithecia. Even when a cross is made by mixing mold of opposite mating types it is necessary for one or the other to produce protoperithecia before fertilization can take place.

After a nucleus has migrated through a trichogyne into the protoperithecium a large number of equational divisions of nuclei of opposite mating types take place side by side with subsequent fusion of pairs. Each fused nucleus then undergoes meiosis to produce 4 haploid nuclei. These undergo two mitotic divisions to give 8 ascospores, each with 2 identical nuclei. Spore pairs are also identical in genetic constitution since the last two divisions are mitotic and since the asco-

spores are contained in a thin-walled sac which holds them in a definite order established by the direction of nuclear segregations during

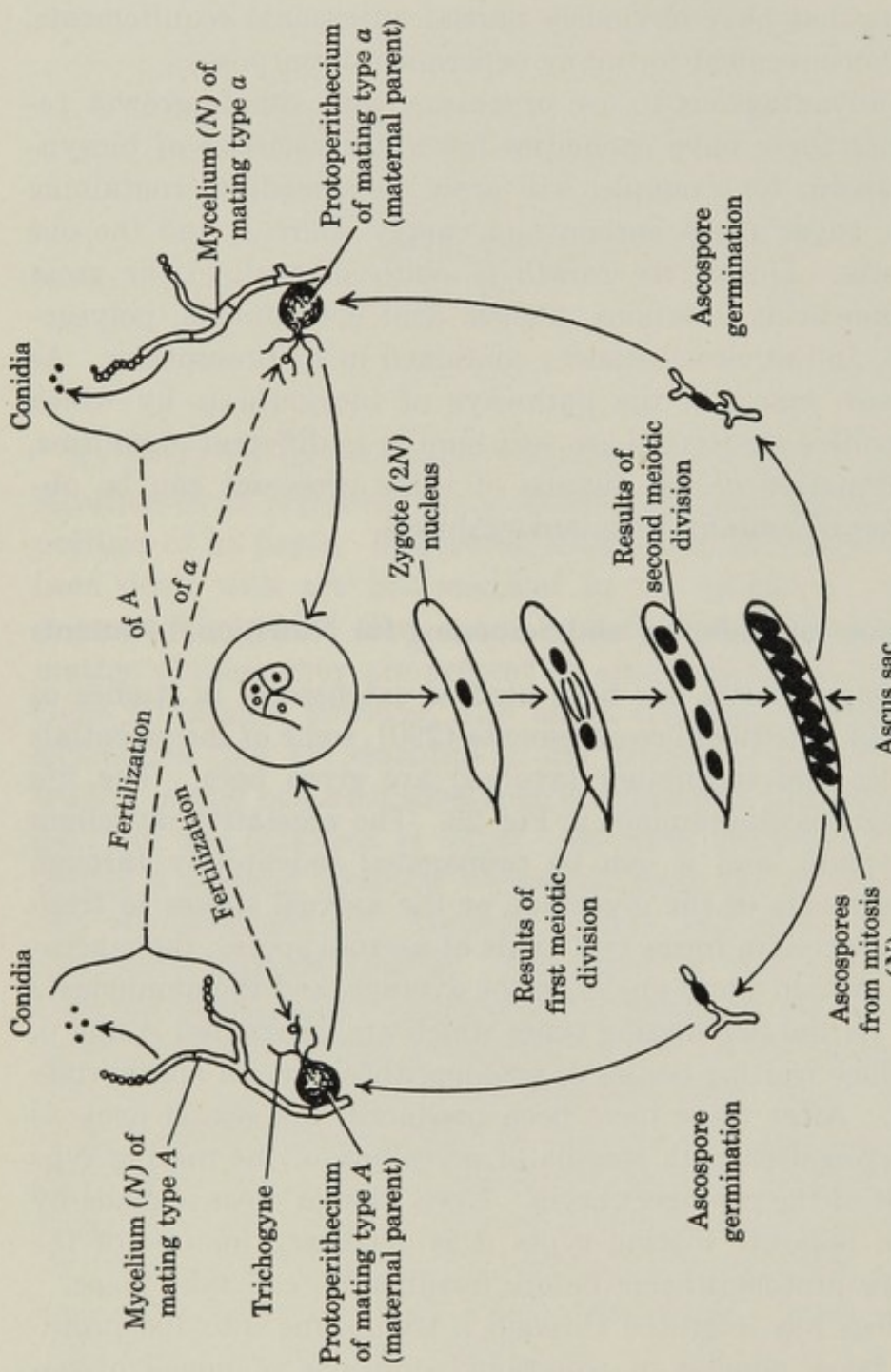


Fig. 28. The life cycle of *Neurospora crassa*. The haploid mycelium reproduces asexually by producing conidia (macro- and micro-) which germinate to give more haploid mycelium, as well as by simple proliferation of the existent mycelium. In this diagram the sexual cycle only is illustrated. Two mating types are required for sexual reproduction. Fertilization takes place by the passage of nuclei of conidia or mycelium into the protoperithecia of the opposite mating type through the trichogynes. Fusion of the nuclei of opposite mating types takes place within the protoperithecia.

meiosis. A perithecium may contain as many as 2 to 300 asci each derived from identical fusion nuclei, and when the perithecia mature these ascospores are ejected rather violently from the ascus sacs. The ascospores require heating at 60°C for 30 minutes to induce germination.

The entire sexual cycle requires from 10 to 15 days for normal strains. Reasonably homogeneous cultures can be obtained directly from ascospores, conidia, or bits of mycelium, but it should be noted that several million nuclear divisions take place during the development of a culture, and there is ample opportunity for the occurrence of spontaneous mutations. Thus, no two cultures, even those derived from identical ascospore pairs, are to be considered as being absolutely identical, although they almost always appear to be by the relatively crude criteria ordinarily used for identification. Obviously, the relative identity of two cultures will diverge in subsequent subcultures, since, even though the mutation rate of any single gene may be low, there are many genes that can mutate. The spontaneous appearance of nutritional mutants is quite rare, and usually the mutations accumulated during subculturing only modify growth characteristics slightly. Still, these spontaneous mutations must always be taken into account in descriptions of phenotypes in *Neurospora* and in other organisms.

Genetic analyses in *Neurospora* can be carried out in two general ways. Whole asci can be removed from nearly mature perithecia and the spores separated in order by hand, using a thin glass needle under a 40 to 80 power magnification. These spores can be immediately transferred to individual tubes, activated, and allowed to produce cultures, or they may be activated directly on an agar-containing petri dish after separation of the spores in order and far enough apart so that phenotypes of the germinated spores can be observed directly under the microscope. The latter procedure is the more rapid, and it is applicable to many nutritional mutants since ascospores from these will germinate but will grow very little without a nutritional supplement. Figure 29 shows germinated ascospores from a cross of a colonial and a nutritional mutant. By isolating and examining a large number of ascospores from single asci in this way centromere distances and relative positions of various genes on the seven chromosomes of *Neurospora* can be estimated.

Genetic analyses can also be carried out, using random spores that have been shot out of mature perithecia. These are plated out, 2–5,000 on minimal agar petri dishes, and the total numbers of different types counted. With suitable markers this is a rapid and useful procedure which permits observations of very large numbers of progeny. An example showing segregation of a colonial and a nutritional mutant among random spores is illustrated by Fig. 29. It should be noted that there is some selection of material for observations in both these procedures. In ascus isolations one selects the most nearly mature ap-

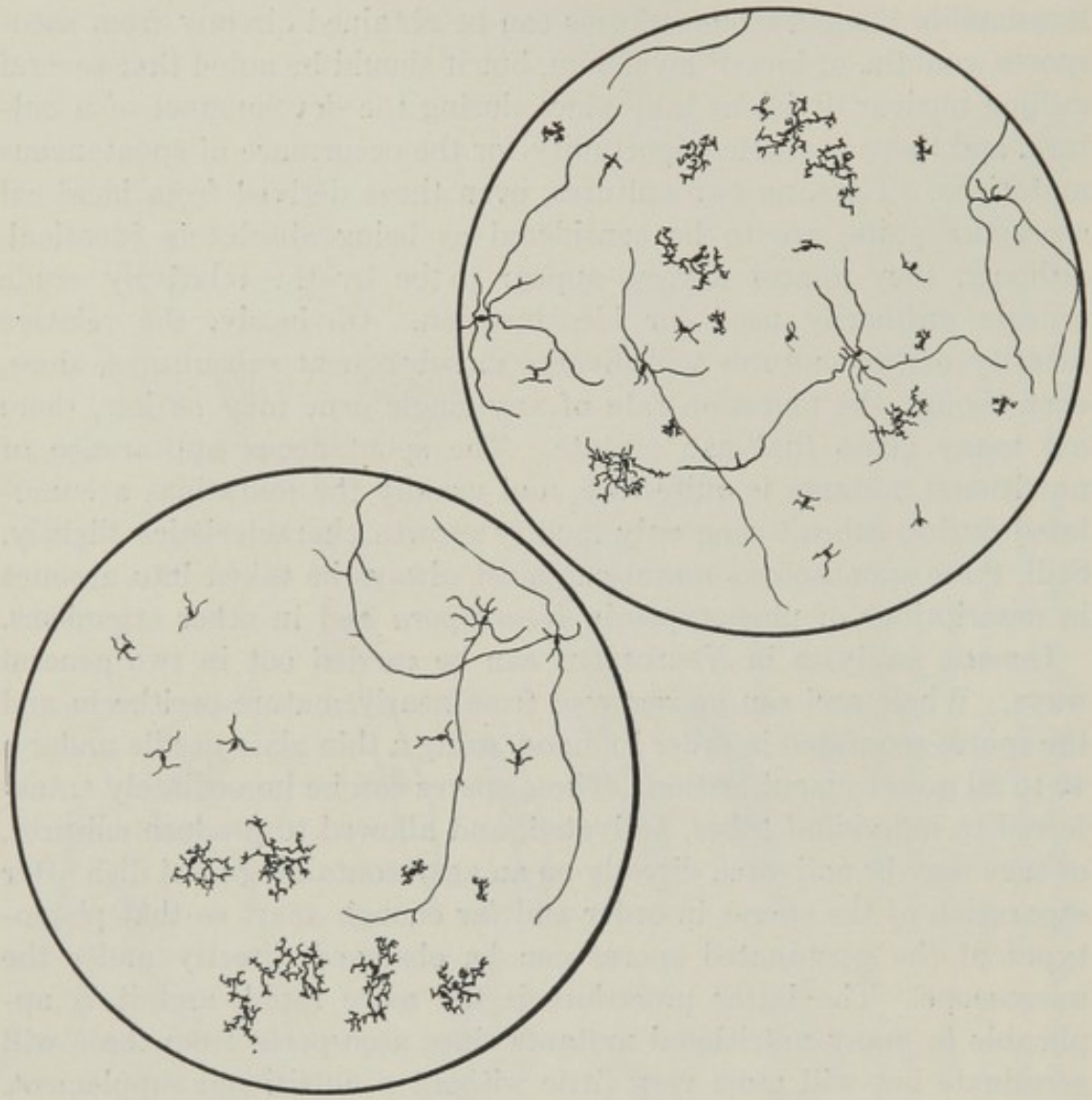


Fig. 29. Germinating Ascospores of *Neurospora crassa*. Camera lucida drawings showing the segregation of two genes in a cross of *Neurospora crassa* mutant 70007 (colonial) and 38502 (pyrimidine). Spores were isolated on minimal agar medium in petri dishes, heated at 60° for 30 minutes for activation, and incubated at 25° for 18 hours. This nutritional mutant (pyrimidine) grows sufficiently on minimal medium to permit identification of the *pyr-co* double mutants.

The figure at the lower left shows segregations in two asci with spore pairs isolated in order. From top to bottom the two asci show, respectively, genotypes *pyr, pyr, co, co* and *wild, pyr, co-pyr, co*. The figure on the right shows results from plating spores from the same cross at random. Genetic analyses can be made directly, using either procedure or by transferring germinated spores to appropriately supplemented culture tubes for further testing. Much larger numbers can be observed conveniently using the random method.

pearing complete asci. Sometimes the maturity is affected by the particular combinations of mutant genes or chromosomal aberrations that are present, while use of random spores assumes that the spores shot out are of equal maturity on a random basis. These two methods frequently give very nearly the same values for map distances between two genes, but extreme selection sometimes occurs. Obviously the random method is more suitable for very close linkages, since it is practicable to observe much larger numbers of progeny by this procedure.

A number of techniques have been developed for the isolation of nutritional mutants of *Neurospora,* as well as other microorganisms, and it is now a simple matter to obtain a great variety in quite a short time. The original method devised by Beadle and Tatum (33) and the procedure of Lein et al. (362) both make use of ascospore isolations. By these procedures conidia from one mating type are treated with a mutagenic agent (see Chapter 3) in a dosage sufficient to produce more than about 90% killing. These conidia are then used to fertilize protoperithecia of the opposite mating type. After mature perithecia are obtained, one spore is removed from each perithecium and transferred to a test tube containing a complete medium (that is, the minimal one which will support growth of wild type plus a complex mixture of metabolites such as are provided by yeast extract, hydrolyzed casein, and nucleic acids). Cultures thus obtained are tested for growth on the minimal medium, and if they do not grow in the absence of supplement they are retained as nutritional mutants.

By the Lein et al. (362) procedure, crosses are made on petri dishes, using treated conidia, and after spores begin to be ejected from the mature protoperithecia (2–5,000 per plate) these plates are inverted for a short interval over plates of agar minimal. The several thousand spores thus collected on the minimal plate are then heat-activated and allowed to germinate and grow for about 20 hours. At this time mutants can be distinguished from wild types as shown in Fig. 29. Those assumed to be mutants are then transferred to a complete medium and tested back on minimal as already described. Specific nutritional requirements of the mutants are then ascertained by systematic tests on minimal medium plus individual pure metabolites such as amino acids or vitamins.

It should be noted that most of the mutants obtained in experiments like these are ordinarily discarded on the basis that they do not fulfill the arbitrarily chosen criteria for desirable nutritional mutants. Some grow too much on the minimal medium; some revert after being subcultured; and many are slow in growth and not sufficiently stimulated

on a complete medium. This selection is shown clearly by data obtained during isolations of nutritional mutants by both of the above methods. For example, using the original method (33), 7,049 ascospores germinated out of 8,795 isolated in one series of experiments. Of these, 54 gave rise to clear-cut nutritional mutants. A much greater number, 489, were distinguishable from wild type because of their slower growth on complete medium. Of these, 159 did not grow sufficiently to permit testing. The remaining 330 were discarded because they did not give sufficiently clear responses to added nutrients.

These same principles undoubtedly apply in procedures for isolating nutritional mutants using direct platings of asexual spores of *Neurospora* as well as in similar methods used in other organisms. With *Neurospora,* nutritional mutants have been obtained directly by plating treated macro- or microconidia on minimal medium containing sorbose to induce colonial growth (640). After wild-type conidia have grown, nutrients are added to the plate and new colonies that appear are isolated. In another adaptation of this method the wild types are permitted to germinate in liquid minimal medium (704). They are then filtered off, the remaining spores are plated out, and mutants are isolated and identified, as described previously. These methods permit testing a very large number of individual conidia, and they are especially useful in selecting strains that have desired nutritional requirements. In this connection it has been observed (362) that quite a number of clear nutritional mutants are inhibited by constituents of complete media, and it is frequently necessary to select for them directly by using minimal medium containing one metabolite as the only adjunct.

2. Types of Nutritional Mutants

Application of these procedures for the isolation of nutritional mutants of *Neurospora* has yielded a great variety with known requirements. These are summarized in Table 13. Data on linkage, compounds accumulated, and references are included in the table.

Some more or less typical growth curves for an amino acid and a vitamin-requiring mutant are shown in Fig. 30. Many mutants grow at nearly a wild-type rate and to about the same total dry weight as the average wild-type strain in the presence of the required metabolite, but a great many do not give normal growth even in the presence of an excess of a required metabolite. Many mutants have been isolated that grow only about 10% as well as the wild type.

Table 13.* Examples of Nutritional Mutants in *Neurospora crassa*

Strain Number	Linkage	Compound Required	Compound Accumulated	Reference
47904	—	*Choline*, dimethylaminoethanol, methionine	Monomethylaminoethanol	297, 288
34486	D	*Choline*, monomethylaminoethanol, dimethylaminoethanol		291, 297, 288
37401	E	*Inositol*		25, 190
4540	A	*Nicotinic acid*	Small quantities α-N-acetyl-kynurenine, 3-hydroxyanthranilic acid	35, 427, 262, 270, 717
3416	A	*Nicotinic acid*	Quinolinic acid	427, 35, 54, 270, 717, 718, 470
E5029	—	*Nicotinic acid*, 3-hydroxyanthranilic acid and 3-hydroxykynurenine	α-N-Acetylkynurenine, kynurenic acid	262
Y31881	—	*Nicotinic acid*, 3-hydroxyanthranilic acid, 3-hydroxykynurenine	α-N-Acetylkynurenine	717
5531	D	*Pantothenic acid*		633, 33, 674
1633		*para-Aminobenzoic* acid		637, 649, 162, 163, 720
7803	D	*Pyridoxin*		619, 33
51602	B	*Riboflavin*		419
18558	A	*Thiamine*	Pyrimidine	635
9185	C	*Thiamine*	Thiazole and pyrimidine	635
32213	—	Any amino acid *except* citrulline threonine, homoserine, serine, lysine, histidine, glycine		178, 179
36703	—	*Arginine*		591, 592
33442	—	*Arginine*, citrulline		589, 590, 592
30837	A	*Arginine*, citrulline, ornithine		591, 592
51077	—	*Arginine*, citrulline, ornithine proline, α-amino-δ-hydroxyvaleric acid		592
30820	—	*Arginine*, citrulline; best when either combined with adenine		589, 590
C84		*Histidine*	Imidazoleglycerol, imidazoleglycerol phosphate, α-ketoisovaleric acid, pyruvic acid	242
51504	A	*Homoserine*		642, 646
16117	E	*Isoleucine and valine*	α-β-Dihydroxy-β-ethyl butyric acid and -β-methyl butyric acid	50, 1, 2
33757	C	*Leucine*		509, 531
15069	A	*Lysine*		214
4545	A	*Lysine*, ε-hydroxynorleucine	Acid labile phosphate, α-ketoisovaleric acid, pyruvic acid	145, 303, 214
33933	E	*Lysine*, α-aminoadipic acid, ε-hydroxynorleucine		304, 214
38706	A	*Methionine*		289
H98	—	*Methionine*, homocysteine	Cystathionine	289, 720
36104	E	*Methionine*, homocysteine, cystathionine		289, 720
9666	D	*Methionine*, homocysteine, cystathionine	Homoserine and threonine	181
86801	—	*Methionine*, homocysteine, cystathionine, cysteine		484

* Linkage data from Houlahan, Beadle, and Calhoun (305).

Table 13. Examples of Nutritional Mutants in *Neurospora crassa* (*Continued*)

Strain Number	Linkage	Compound Required	Compound Accumulated	Reference
80702	—	*Methionine*, homocysteine, cystathionine, cysteine, cysteine sulfinic acid		484
35001	—	*Methionine*, homocysteine, cystathionine, cysteine, cysteine sulfinic acid, cysteic acid		484
8043M	—	*Methionine*, *p*-aminobenzoic acid		622
E5212	—	*Phenylalanine*		262
21863	C	*Proline*		530, 592
H605	C	*Serine*, glycine		307
Y5015	—	*Serine*, glycine, glycolic acid, glyoxylic acid		705
T3207	—	*Serine*, formate, formaldehyde		260
35423	—	*Threonine*		642
44104	—	*Threonine*, α-aminobutyric acid, isoleucine		642
C83	—	*Tryptophan*	Indole-like compound, anthranilic acid	423, 262, 470
10575	C	*Tryptophan*, indole	Anthranilic acid	35, 641, 423, 262, 717, 470
40008		*Tryptophan*, anthranilic acid, indole		459
75001	—	*Tryptophan*, anthranilic acid, indole, kynurenine		717, 470
39401	—	*Tryptophan*, *indole*, kynurenine, 3-hydroxyanthranilic acid, nicotinic acid	Acid labile phosphate	427, 303, 55, 717, 470
C86	—	*Tryptophan*, tyrosine, cinnamic acid, anthranilic acid, indole, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, nicotinic acid, quinic acid, phenylalanine		262, 217, 358
35203	A	*Adenine*, hypoxanthine	Purple pigment	420, 424
37301	D	*Uracil*, cytidine	Acid labile phosphate, pyruvic acid	302, 303, 421, 428
38502	D	*Uracil*, cytidine	Orotic acid, orotidine	302, 304, 303, 421, 428
263	D	*Uracil*, cytidine, orotic acid	Pyruvic acid	302, 304, 421, 428
S-210	—	*Acetic acid*, myristic acid, linoleic acid, linolenic acid	Acetylmethylcarbinol	360, 361, 620, 621
Y-2492	—	*Acetic acid*, *ethyl alcohol*		466
S11	—	*Unsaturated fatty acids*, oleic, linoleic, and linolenic acids		359
37602	A	*Succinic acid*, fumaric acid, malic acid, α-ketoglutaric acid, oxalosuccinic acid, acetoacetic acid, acetic acid, glutamic acid, aspartic acid		374
C24	—	*Formate*, formaldehyde, adenine + methionine		260
A16	—	*Nitrite*, *ammonia*, unable to use nitrate		134
UV392	—	*Ammonia*, unable to use nitrite, nitrate		134

As discussed in more detail in Chapter 8, the acquisition of a nutritional requirement is accompanied by a variety of physiological changes. A very common one is reduced viability. Many of the *Neurospora* mutants will not produce ascospores in self-crosses, and there is a great variation in the time of maturity of perithecia. Most

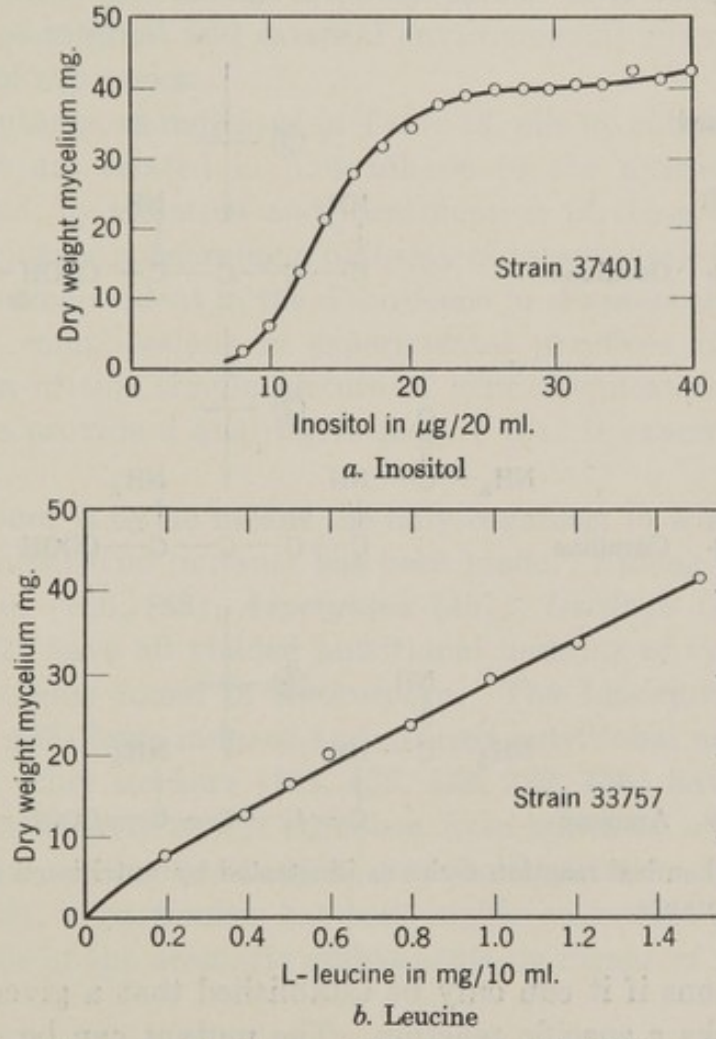


Fig. 30. Growth curves for two nutritional mutants of *Neurospora*. *a*. Inositol. After Beadle (25). *b*. Leucine. After Regnery (509).

of the mutants listed in Table 13 have, however, been subjected to some genetic analysis, and so far as the data go the nutritional requirements indicated can be associated with the mutation of a single gene. It should be emphasized, nevertheless, that any one of them may carry mutations of closely linked genes, and in many cases the data are insufficient to eliminate the possibility of double mutants with genes as far apart as one or two map units.

The discussions in the previous chapter on pigment inheritance have already anticipated the possibility of using information derived from

studies of inherited chemical differences in deducing something of the processes of biosynthesis. The nutritional mutants of microorganisms have proved especially useful in this regard, as is clearly shown in the discussions of metabolic patterns in Chapter 8. The principles involved in experiments directed to this end are quite simple. Often it is not necessary to know the mechanism by which genes control bio-

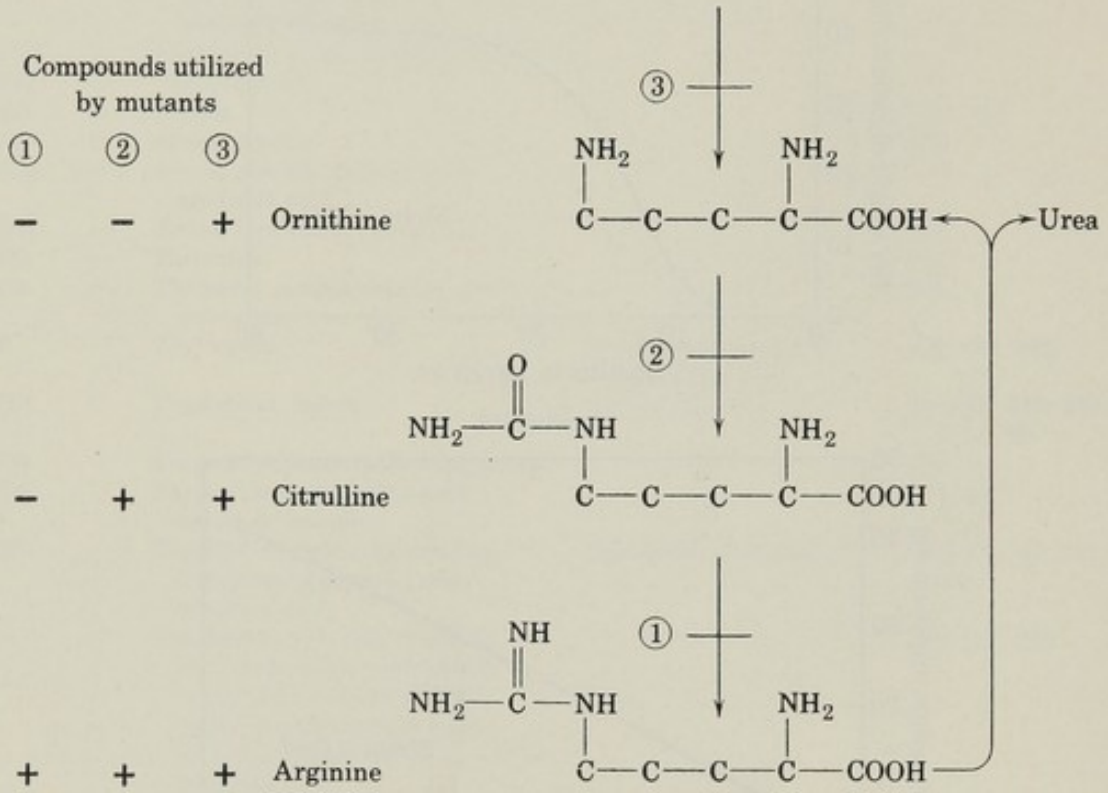


Fig. 31. A biochemical reaction series as illustrated by nutritional requirements of *Neurospora* mutants.

chemical reactions if it can only be established that a given mutation effectively blocks a specific reaction. The mutant can be used in the same way specific inhibitors are used.

In anticipation of much more detailed discussions, consider the example of the series of arginine-requiring mutants of *Neurospora,* indicated in Fig. 31. Of three genetically different mutants (3) will utilize ornithine, citrulline, or arginine; (2) will use citrulline or arginine; and (1) will use only arginine. These facts suggest that arginine can be produced from ornithine *in vivo* through a linear series of reactions as shown in Fig. 31. Thus, it is considered that the genetically different mutants which will utilize the same metabolite frequently have effective blocks at different steps in biochemical reaction series. This is not necessarily always true, since each step is chemically com-

plex and each can very likely be blocked in a number of different ways. Nevertheless the over-all principle is sound, as has been established by investigations of many reaction series. By this principle nutritional mutants will utilize intermediate compounds that come after the block and not those that come before it. This is an idealized situation, and very frequently the blocks are only partial in nature and subject to a variety of internal and external environmental changes of the completeness of expression.

Some mutants, as indicated in Table 13, pile up substances before the block that are related in biosynthesis to the nutrient required for growth, and, by isolation and identification of these, further circumstantial evidence concerning a pathway of biosynthesis can be obtained. As will become evident in the discussions in chapters that follow, there are many complications in experimental practices that involve the application of this simple picture of gene-chemical reaction relation, but it does provide a qualitative picture that is experimentally useful (290, 293).

Neurospora is by no means the only organism in which an intensive study of nutritional mutants has been made. Among the other fungi, *Ophiostoma* (186, 188), *Aspergillus* (491), *Ustilago* (482), and *Penicillium* (52) have all yielded nutritional mutants of types identical or related to those found in *Neurospora*. The Lindegrens and Pomper (379, 380, 488) have induced and utilized nutritional mutants in yeast, and many other workers (131, 132, 133, 223, 356) have found *E. coli* to be a particularly useful organism from which to obtain nutritional mutants for use in the study of metabolic patterns (see Chapter 8). Davis (133), in particular, has been highly successful in analyzing the biosynthesis of the aromatic amino acids by means of *E. coli* mutants.

3. General Comments

The experimental results discussed in this chapter and the preceding one provide a variety of examples which demonstrate that one effect of gene mutation is to produce changes in cellular chemical composition in specific ways and in varying degrees. It is apparent that this comes about by causing changes in the rates of conversion of chemical substances into other chemical substances, and frequently it appears that a specific gene corresponds in its action to a specific reaction. Such a simple direct relation does not mean that the mechanism of control is also simple and direct. The chemical composition of the cell is in general the over-all result of the relative rates of reaction,

and a genetic block merely shifts the intensity of reactions into different channels. Whenever relative reaction rates permit accumulation of some substance in noticeable quantities, then a characteristic chemical composition becomes manifest. These comments anticipate more detailed discussions in other chapters. They are made here to emphasize that it is not just chemical differences that are inherited, but it is the processes which result in the chemical composition of the cells of the individual.

CHAPTER 6

Some Problems of Biochemistry

Within the limits of sensitivity of chemical and physical analytical methods it is possible to arrive at reasonably precise descriptions of many chemical differences that result from changes in the genetic constitution of an organism. Several correlations of this kind have been discussed in Chapters 4 and 5. This analytical approach has yielded a tremendous amount of essential information, but even when it becomes practicable to make accurate determinations of the more complex chemical components of cells, as well as of the simpler ones that are now known, this knowledge of composition cannot, by itself, provide an understanding of the problem of genetic control of biochemical constitution. It is necessary in addition to have a knowledge of the mechanisms and the rates of interconversions of cellular substances. Actually it is this dynamic aspect of biochemistry that has the more fundamental significance, since it can be considered that the chemical composition of cells is only a result of a complex interaction of biochemical reactions. In general, cellular components that exist in definite concentrations do so because of a balance between the rates of the reactions by which they are formed and the rates of the reactions by which they are transformed into other substances. Under constant environmental conditions, reaction rates and thus chemical composition should remain constant in any system that is already balanced; consequently such a system can be said to be operating under conditions of a steady state. It is obvious that in such a balanced system even an infinitesimal change in the rate of any one reaction must result in a readjustment of the entire system.

There is ample experimental evidence, as pointed out long ago by Goldschmidt (205) and Wright (707), to support the contention that gene mutations result in alterations in rates of biochemical reactions, and it is therefore essential to understand the factors that influence

the relative reaction rates. Unfortunately the science of biochemistry has not advanced to the point where it can supply all the necessary information for this purpose. Still, all the information that it can furnish should be considered carefully in an examination of the problem of the genetic control of metabolism. The material in this and several following chapters represents an attempt to select descriptive details that seem particularly pertinent to the problem. The idea of the alteration of metabolism through large or small changes in a reaction rate should be borne in mind throughout the discussion.

1. Reaction Rates

Some Properties of Enzymes

The known biochemical catalysts or enzymes are proteins, or proteins conjungated with other substances of varying complexity. They are produced in all living cells. Over 50 have been isolated in a crystalline form, and several times this number have been shown to exist. The enzymes have molecular weights lying in the range of approximately 20,000 to 1,000,000 or more, and many dissolve in water or dilute saline, giving colloidal solutions. Owing to the great size and accompanying colloidal properties of the enzymes it is not yet possible to devise criteria by which they can be described as pure chemicals with known compositions and spatial configurations. It is quite clear that crystallinity is not adequate as a criterion of purity. Nevertheless, a sufficient stage of purity has been attained with a number of enzymes so that it is certain that the catalytic activity is indeed due to properties of the large molecules and not to adsorbed impurities. Still, most enzymes can be recognized only by their activities.

It is generally considered that an enzyme neither initiates a reaction nor provides energy for inducing a chemical change. It may and often does transfer energy, but the specific reaction that it catalyzes must be thermodynamically possible (see p. 138, coupled reactions). Enzymes then merely increase the rates at which spontaneous reactions take place and this increase can be enormous under physiological conditions. The action of an enzyme in the control of a reaction rate is influenced by a variety of environmental factors, a good many of which exert their influences by changing the effective concentration of the enzyme itself. Some of these important variables are listed below.

1. Concentration of an enzyme.
2. Concentration of a substrate or substrates.

3. Concentration of inhibitors (including reaction products).
4. Concentration of various ions (such as hydrogen ions).
5. Temperature.
6. Oxidation-reduction potential of the environment.

The two variables temperature and *p*H have been used very extensively in enzymology as criteria for the characterization of enzymes. However, even with highly purified systems, experimentally determined *p*H and temperature optima values depend appreciably on the experimental methods used in their evaluation. This is because an observed reaction rate is often a summation of a true reaction rate, and a rate influenced by a complication due to enzyme denaturation. With partially purified systems or *in vivo* systems these errors can be large and very complex in nature because of the stabilizing or denaturing actions of substances in the chemical environment. Furthermore, the properties of an enzyme *in vivo* need not be identical with, or even very similar to, the properties of the same enzyme when purified. However, it is expected that the enzyme will be influenced by the same variables but not necessarily to the same extent in different environments.

Enzymes in general are unstable substances even under physiological conditions where extremes of *p*H values and high temperatures and salt concentrations do not ordinarily exist. When such extremes are normal, as in the strong acidity of the stomach or in the environments of organisms that live at elevated temperatures, the intrinsic properties of the enzymes are such as to give them a relative stability to the extreme conditions. One other significant property of some enzymes is that their stability to various environmental changes can be increased by the presence of the substrates upon which the enzymes act (336, 401). Many workers have found that the addition of substrates improves yields in the isolation of a variety of enzymes, but not many systematic studies of the protective effects of substrates have been made. Such a protective action may be of great importance in maintaining a balanced pattern of enzymes in a cell or in permitting the accumulation of an enzyme in an adaptive system (see p. 306).

The foregoing general discussion of the properties of enzymes is necessarily brief, and the reader is referred to the extensive literature on the subject for further details (360, 345). It is now important to examine some of the quantitative aspects of the reaction rates of enzyme catalyzed systems. As already pointed out, quantitative relations as determined for isolated enzymes probably are not valid for a description of the action of the same enzymes *in vivo*. Nevertheless,

the same influencing factors function under both conditions, and an examination of the quantitative aspects of isolated enzyme-catalyzed reactions yields a great deal of information that is of at least qualitative importance in understanding the properties of *in vivo* enzyme systems. Some of the significant points of quantitative enzymology are, therefore, summarized in the following sections.

Estimation of Reaction Rates

Consider a simple isolated system containing a pure enzyme and a pure substrate dissolved in water or buffer. For a hydrolytic reaction the following equation can be written:

$$\underset{\text{(Substrate, } S_f\text{)}}{ROR' + H_2O} \xrightarrow[E]{} \underset{\text{(Enzyme, } E_f\text{)}}{ROH} + \underset{\text{(Products, } P\text{)}}{R'OH}$$

Since the total concentration of H_2O does not change significantly during the reaction this component can be neglected. It will be assumed that the reaction proceeds by formation of a complex between the substrate and the enzyme and that the complex can decompose to re-form the reactants or to form enzyme plus products in accordance with the following equation.

$$(1) \qquad S_f + E_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P$$

The subscript f refers to the free (uncombined substances), and k_1, k_2, and k_3 are reaction rate constants. By application of the mass law and several reasonable assumptions Michaelis and Menten (411) derived an equation which relates the concentration of the substrate (S) and the reaction velocity, v, for reaction 1:

$$(2) \qquad v = \frac{V_m(S)}{K_m + (S)}$$

Here the apparent dissociation constant $K_m = k_2/k_1 = (E_f)\,(S_f)/(ES)$, and it is numerically equal to (S) at half maximum velocity. The maximum velocity, V_m, is a theoretical value approached by v at large substrate concentrations. Under these conditions an enzyme molecule is surrounded by substrate molecules, and with every dissociation of the complex ES a new substrate molecule is immediately ready to enter into combination. Although an enzyme in such a

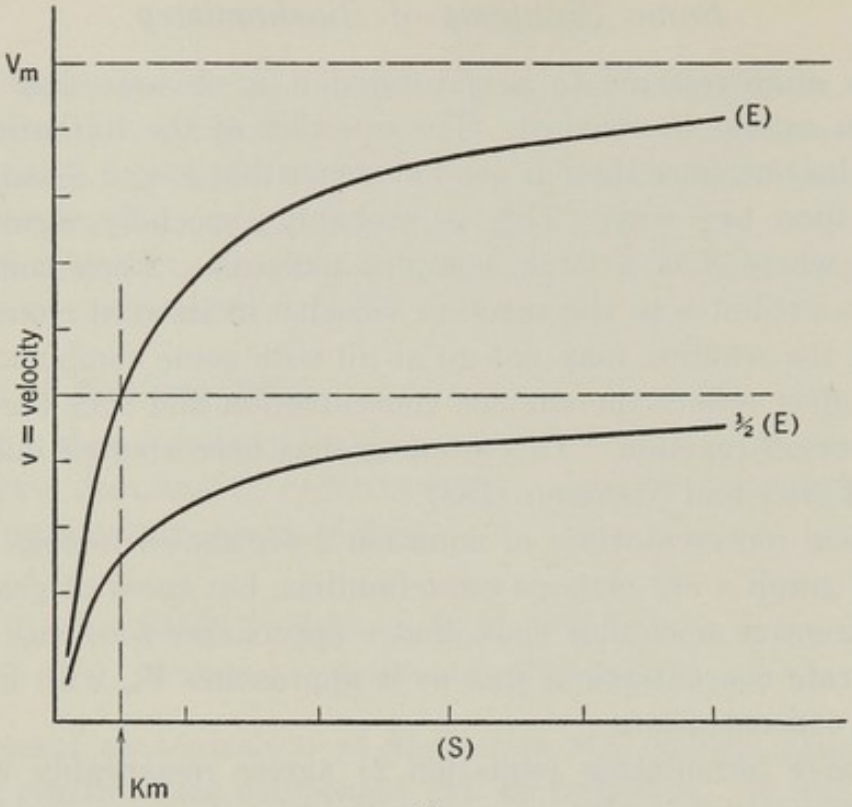
system is often referred to as saturated it is obvious that complete saturation cannot be reached. The question of the formation of ES is a complex one since there is good evidence that E and S can combine in more than one way. This is probably especially significant in reactions where S is a large, complex molecule. Each combination possible contributes to the reaction velocity in its own characteristic way; i.e., the reaction may not go at all with some combinations and these in effect reduce the enzyme concentration and thus the velocity of the over-all reaction. This situation has been treated mathematically by Foster and Niemann (183).

Graphical representations of equation 2 are shown in Fig. 32. The curves of graph *a* are perhaps most familiar, but those of graph *b* are more instructive since they show that v approaches zero with decreasing substrate concentrations just as it approaches V_m with increasing substrate concentrations.

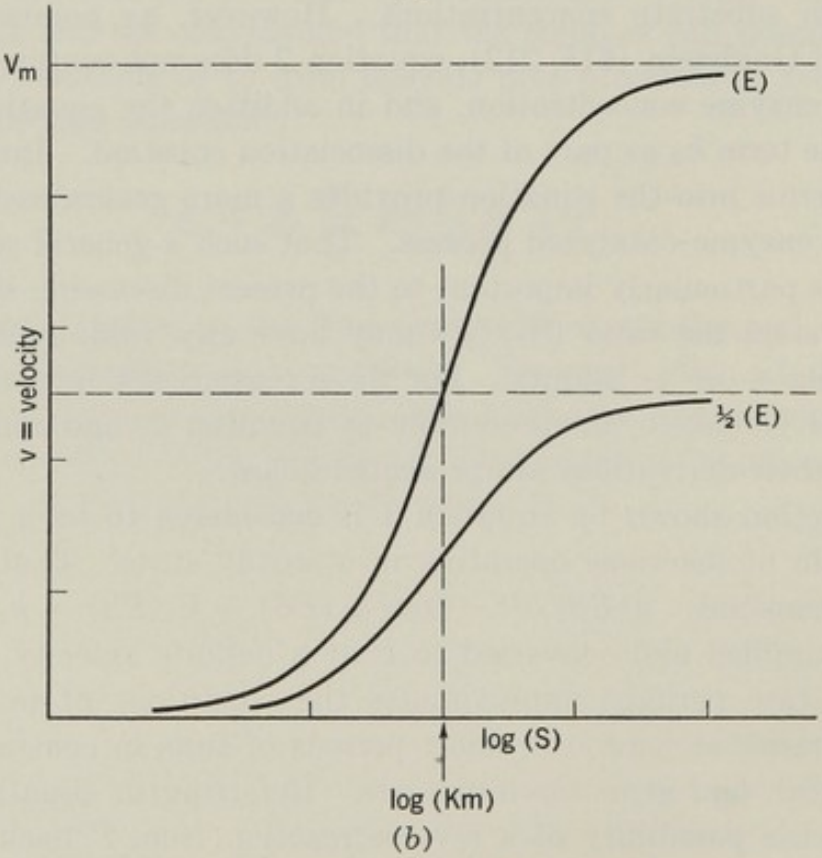
The above formulation (equation 2) agrees remarkably well with many experimental results obtained with isolated enzymes and at relatively high substrate concentrations. However, as pointed out by Straus and Goldstein (618, 212), equation 2 does not contain the term (E_f), the enzyme concentration, and in addition the equation should contain the term k_3 as part of the dissociation constant. Introduction of these terms into the equation provides a more generalized description of an enzyme-catalyzed process. That such a general relation be obtained is particularly important to the present discussion since in an *in vivo* system the ratio $(S)/(E)$ may have any value within limits approaching + or − infinity. For these reasons the formulations of Straus and Goldstein are preferable to equation 2, and some of the details of their derivations are presented below.

The reaction shown by equation 1 is considered to be a part of a whole chain of reactions operating in a steady state. That is, (ES) remains constant, $d(ES)/dt = 0 = k_2(ES) + k_3(ES) - k_1(S_f)(E_f)$, and S is supplied and converted to P at a definite velocity, v. Such a steady state perhaps approximates the conditions of *in vivo* enzyme reactions, at least over short periods of time in constant physical, chemical, and genic environments. Referring to equation 1 and neglecting the possibility of a reverse reaction from P back to S the constant,

$$K_s = \frac{(E_f)(S_f)}{(ES)} = \frac{k_2 + k_3}{k_1} \tag{3}$$



(*a*)



(*b*)

Fig. 32. Graphical representations of the Michaelis-Menten equation. (*a*) Plot of reaction velocity vs. substrate concentration (*S*) showing $K_s = (S)$ at $v = \frac{V_m}{2}$. (*b*) Plot of reaction velocity vs. log (*S*) showing that the velocity approaches limits at both ends of the curves.

and

$$(E_{\text{total}}) = (E) = (E_f) + (ES) \tag{4}$$

while

$$(S_{\text{total}}) = (S) = (S_f) + (ES) \tag{5}$$

As noted previously,

$$v = k_3(ES)$$

and

$$V_{\max} = k_3(E_{\text{total}})$$

since $(E_{\text{total}}) = (ES)$ at maximum velocity.

Now, for convenience, by definition the fractional activity

$$a = \frac{v}{V_m} = \frac{(ES)}{(E)} \quad \text{and thus} \quad (ES) = a(E) \tag{6}$$

Substitution of the equivalents of (E_f), (S_f), and (ES) from equations 4, 5, and 6 respectively into equation 3 yields the following equality:

$$(S) = K_s \frac{a}{1 - a} + a(E) \tag{7}$$

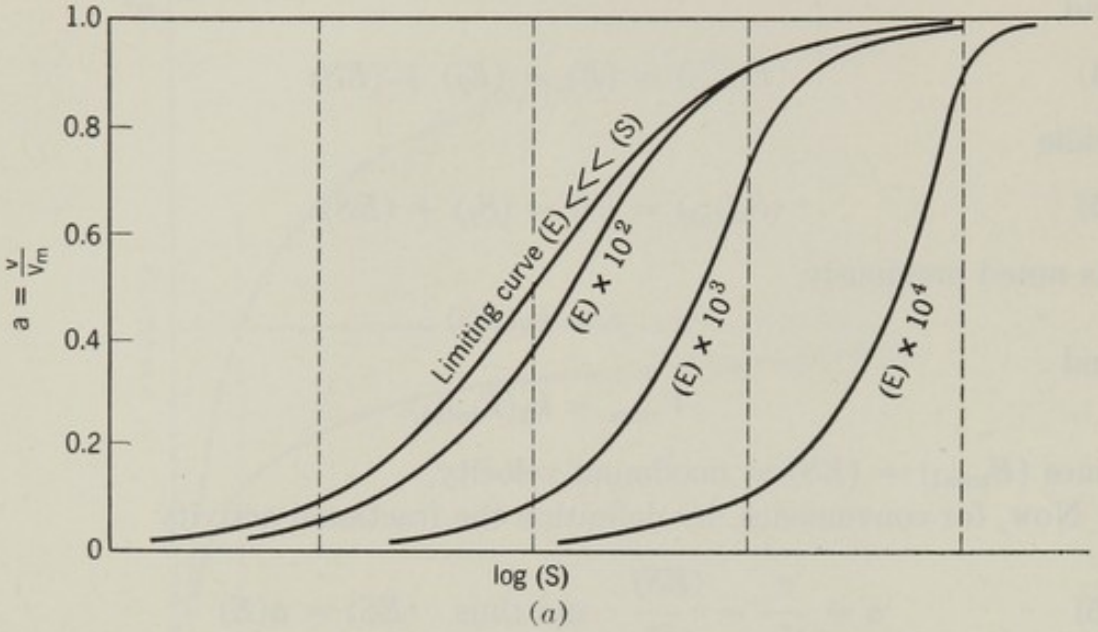
In order to simplify equation 7 further, for practical manipulations, Straus and Goldstein introduced the idea of specific concentrations which, like specific gravities, are dimensionless. The specific concentration of E is $E_s' = (E)/(K_s)$, and that of S is $S' = (S)/K_s$. Substitution in (7) gives the dimensionless general equation

$$S' = \frac{a}{1 - a} + aE_s' \tag{8}$$

Equation 7 or 8 can of course be expanded by substitution of equalities shown above and written:

$$(S) = K_s \frac{v/V_m}{(1 - [v/V_m])} + \frac{v}{V_m}(E) \tag{9}$$

If (E) is assumed to be negligible relative to (S) then the second term can be dropped and rearrangement gives the familiar Michaelis and Menten equation 2. It is at once evident from equation 9 that a reaction velocity v is dependent simultaneously on the concentration of the substrate, the concentration of the enzyme, and the properties of the enzyme described by the constants K_s and V_m. These two con-



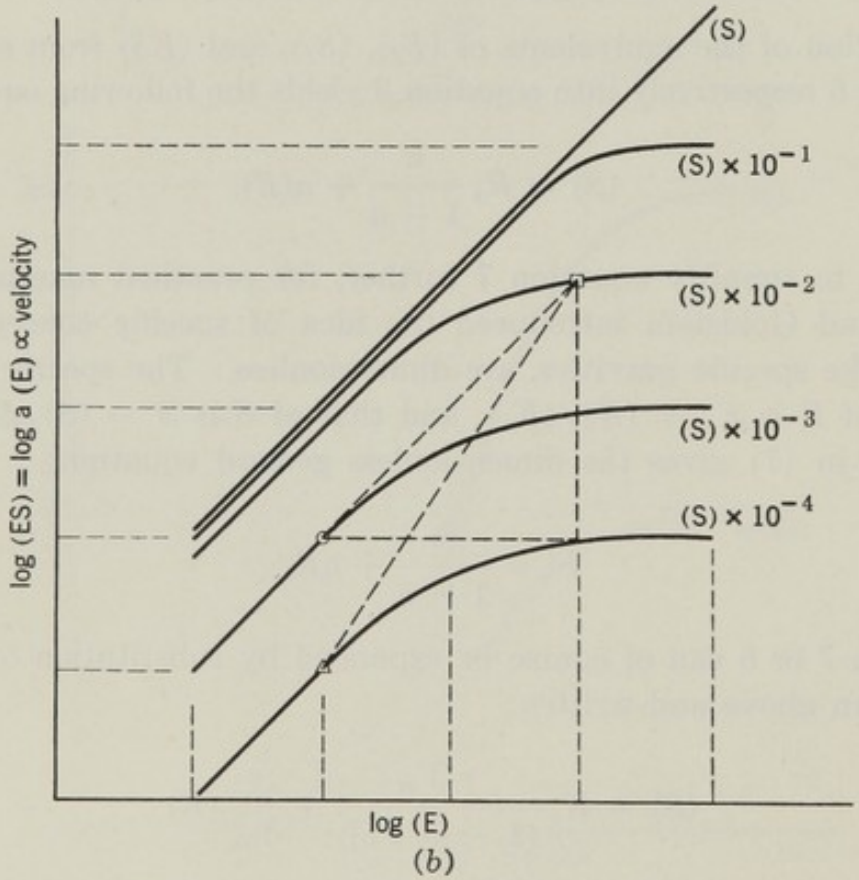


Fig. 33. Graphical representations of the Straus-Goldstein equation. (*a*) Plots of velocity vs. log (*S*) at different relative concentrations of *E*. The limiting curve at the left where (*E*) is much smaller than (*S*) corresponds to the curves in Fig. 32*b*. (*b*) Plots of log velocity vs. log (*E*) at different concentrations of *S*. The symbols, ⊡ = initial state of a system, ⊙ = expected state by dilution of the system 100 fold, and △ = the actual state of the system after dilution, illustrate the fact that the magnitude of the effect of an imposed change is dependent

stants are evidently related to the "affinity" of the enzyme for the substrate and the rapidity with which the enzyme is able to convert the substrate to the products. The equivalent equations 7, 8, and 9 are represented graphically in Fig. 33, parts *a* and *b*. The limiting curve at the left in Fig. 33*a*, where (E) is much smaller than (S), is essentially the same as that obtained from the Michaelis and Menten equation and shown in Fig. 32. In this region the fractional activity is independent of the enzyme concentration. The series of curves to the right show an increasing dependence of the fractional activity on (E) until $a = (S)/(E)$ and the curves become nearly identical in shape and are equally spaced with equal changes in log (E) or (S). The curves of Fig. 33*b* illustrate clearly the relations existing between (E), (S), and the rates at which products are formed. It should be observed that a tenfold change in (E) or in (S) can result in a change in the reaction velocity of any amount from zero to tenfold depending on the state of the operating system prior to the change. A related property of such an enzyme system, the dilution effect, is also illustrated in Fig. 33*b*. As shown in the figure, if an enzyme system operating under conditions shown by the square is diluted a hundredfold, the reaction velocity will decrease, not a hundredfold as might be expected, but nearly a thousandfold as shown in the figure. On the other hand, with an initial high (S) instead of $(S) \times 10^{-2}$, the velocity change would be proportional to the dilution.

The foregoing discussion has been concerned with an analysis of the simplest kind of an enzyme-catalyzed system, but it serves as an adequate illustrate of principles that are certainly quantitatively applicable to somewhat more complex systems and probably qualitatively applicable to *in vivo* biochemical reactions. *Particular note should be made of the fact that the magnitude of a biochemical reaction depends not only on the magnitude of a change in concentration of a component that enters into the reaction but also on the existing operational state of the system.* The dilution effect (p. 118) serves as a good example of this important point. That is, if the cytoplasm of a living cell can become diluted (or concentrated) with respect to water it is expected that some reaction rates will be altered in proportion to the dilution while others will not. Which result is obtained depends on the relative concentrations and specific properties of the components that

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on the pre-change state of the system. In the illustration, if the initial state had been at (S) instead of $(S) \times 10^{-2}$, then the reaction velocity change would have been proportional to the dilution.

enter into each reaction. For these reasons a change as simple as a dilution requires extensive revisions in an existing balance of reactions and concentrations of cellular components. These conclusions are necessarily qualitative in nature since they are based on observations of simple isolated systems. Still, they should be considered along with the discussion of more complex systems on the pages that follow.

Enzyme Inhibitors and Inhibition

The inhibition of enzyme-catalyzed reactions is a very common and well-known phenomenon that has been used extensively in describing the action of enzymes as well as in investigations of enzymatic reactions in complex systems and whole tissues. Inorganic ions, such as F^-, CN^-, N_3^-, $S^=$, $AsO_4^=$, and heavy metal cations (Cu^{++}, Ag^+, and Hg^{++}) owe at least one part of their toxicities to inhibition of enzyme systems. Similarly a great number of organic substances (e.g., iodoacetate, malonate, various alkaloids, etc.) act as inhibitors for both *in vitro* and *in vivo* enzyme systems. This field of endeavor has expanded enormously following the suggestion of Fildes (703) and Woods (702) that sulfonamides exert their inhibitory actions because of their chemical structural relations to that of the B vitamin, *p*-aminobenzoic acid. That is, the drug competes with the vitamin for combination with an enzyme, the action of which is essential for growth of the cells that are inhibited by the drug. As a consequence of these observations a very large number of analogs of normal metabolites have been prepared and tested as inhibitors and chemotherapeutic agents. There can be little doubt of the validity of the principle involved, i.e., that structurally related substances do act as antagonists for specific actions of enzymes. On the other hand it has not been possible to predict the structural changes that can be made in a normal metabolite in order to produce a good antagonist.

These substances mentioned above, for the most part, do not occur or function as inhibitors in living systems, but it is most important to note that a very large number of inhibitors do occur naturally. Actually it is probable that nearly every substance produced in an organism can act as an inhibitor for the same, or another, organism under some set of conditions. Attention is usually focused on the more highly active inhibiting compounds such as the antibiotics, the regulatory hormones, and antibodies or other specific protective agents.

Some good examples of inhibitors of the last type are to be found in the work on the isolation from pancreas tissue of a protein that interferes with the action of trypsin (457) and in the early work on

the intestinal parasitic roundworm, *Ascaris*. It has been found that extracts of the worm inhibit the action of the pepsin and trypsin that are found in the intestinal tract of the host. The extracts do not, however, inhibit the action of the proteolytic enzyme papain, and the living worms can be digested with this enzyme, which is of plant rather than of animal origin. The naturally occurring inhibitors such as the specific ones mentioned above are undoubtedly extremely important in the processes of metabolic control, and some of them will receive attention in the subsequent discussions. However, it is more appropriate to consider the phenomenon of multiple substrate inhibitions.

It is well known that many enzymes catalyze a type of reaction, and thus they act on a variety of naturally occurring substances that frequently occur together in the same tissue. Amylases, phosphatases, proteolytic enzymes, transaminases, hexokinases, and many other enzymes can be cited as examples. Where two or more substrates exist together in the presence of an enzyme that will act on both, each substrate can act as an inhibitor for the reaction involving the other. A good illustration of these facts comes from the work of Foster and Niemann (182), who studied the kinetics of the simultaneous hydrolysis of acetyl-L-tryptophanamide and acetyl-L-tyrosinamide in the presence of crystalline chymotrypsin. The reactions involved are indicated below.

$$\begin{array}{ccc} \text{Acetyl-L-tryptophanamide} & \rightarrow & \text{Acetyl-L-tryptophan} + NH_3 \\ & \nearrow & \\ \text{Chymotrypsin} & & \\ & \searrow & \\ \text{Acetyl-L-tyrosinamide} & \rightarrow & \text{Acetyl-L-tyrosine} + NH_3 \end{array}$$

Here the two substrates appear to combine with the enzyme at the same site, and there is a quantitative dependence of each reaction velocity on the relative concentration of the two substrates and their relative affinities for the enzyme. (Some aspects of inhibition kinetics are considered in the next section.)

It is not known to what extent multiple substrate inhibitions contribute to the normal balance in metabolism, but there is reason to believe that such phenomena do occur *in vivo* and that they account for the regulation of the velocities of many reactions.

Inhibition Kinetics

It is appropriate at this point to examine some of the quantitative aspects of the action of inhibitors on reaction velocities of relatively

simple enzyme-catalyzed reactions. Reviews by Wilson (697) and McElroy (392) provide many details that will not be presented here. Inhibitors have been classified into three general categories of simple isolated systems.

1. Competitive: The inhibitor competes with the substrate for a specific active site on the enzyme.

$$E + S \rightarrow ES$$

$$E + I \rightarrow EI$$

2. Non-competitive: The inhibitor and the substrate combine with the enzyme independently at different positions on the enzyme surface.

$$S + E + I \rightarrow S{-}E{-}I$$

3. Uncompetitive: The inhibitor combines with the enzyme substrate complex but not with either of these components alone.

$$ES + I \rightarrow \begin{matrix} & I & \\ E & & S \end{matrix}$$

These somewhat arbitrary classifications do not delineate all of the possibilities, but they do describe phenomena that are consistent with a good deal of experimental data. Again, referring to Goldstein's general formulation and to competitive inhibition only, we must consider simultaneously the concentrations of the substrate (S), the enzyme (E), and the inhibitor (I). Thus, as in the derivation of equation 8,

$$K_s = \frac{(E_f)(S_f)}{(ES)}$$

and, in addition,

$$K_I = \frac{(E_f)(I_f)}{(EI)}$$

By combining these two equations through the common term (E_f) and by appropriate substitutions as described previously the following general equation can be obtained

(10)

$$I' = \left[(S' - aE_s') \left(\frac{1 - a}{a} \right) - 1 \right] + \left[(1 - a) \left(1 + \frac{1}{S' - aE_s'} \right) \right] E_I'$$

Here $I' = (I)/K_I$; $S' = (S)/K_S$; $a = v/V_m$; $E_S' = (E)/K_s$, and $E_I' = (E)/K_I$, and the equation describes the quantitative relation be-

tween E, S, K_S, K_I, v, and V_m. In experimental practice the equation can be simplified by setting up conditions such that some of the terms become negligible. For example, in a system with an excess of inhibitors but not of substrate the equation becomes

$$I' = \left[(S' - aE_S') \left(\frac{1 - a}{a} \right) \right] - 1$$

At another extreme where the enzyme is present in great excess over both the substrate and the inhibitor equation 10 can be reduced to

$$I' = (1 - a)E_I'.$$

Under these circumstances there is more than enough enzyme to combine with all of the substrate and inhibitor, and competitive inhibition does not exist.

The general equation 10 which describes competitive inhibition over a broad range of values for the variables it contains is analogous to the general equation 8 where (I) and K_I are not included. The additional complexities resulting from the introduction of this variable and constant do not alter the principle already set forth, i.e., that the magnitude of a change of a reaction velocity is dependent not only on the magnitude of change of one of the variables (S), (I), or (E) but also upon the specific pre-existing relations between these variables and the constants K_s and K_I. The dilution effect and other quantitative aspects of the problem have been discussed by Goldstein and they will not be elaborated here since many *in vivo* systems are too complicated to be analyzed quantitatively by use of equation 10. For example, some reactions involve the interaction of a number of additional components, as indicated:

$$\left.\begin{array}{l} S_{1,2\cdots} \\ I_{1,2\cdots} \\ \text{Coenzyme} \\ \text{Metal ion} \end{array}\right\} + E \rightleftharpoons \begin{array}{c} I \\ | \\ Me\text{—}E\text{—}S \\ | \\ Co \end{array} \leftrightharpoons E + P_x + Co + Me + I$$

In this example each of the reactants will have some characteristic affinity for the enzyme as in the simplified case. That some reactions are probably this complex does not necessarily introduce any new principles or ideas. It is clear, however, that the variables and combinations of variables that can influence the reaction velocity will be enormously increased. That is, the reaction velocity can become altered in many more different ways than in the simple systems.

2. Enzyme Complexes

The state of an enzyme itself is obviously very important in the determination of reaction rates. Experimental evidence has shown that cells contain organized bodies in addition to the nucleus and that a good many enzymes are concentrated within these cellular particles. Furthermore, it seems highly probable that the enzymes are oriented in an organized fashion within these complexes. Thus, it is apparent that there exists in cells a morphology at a molecular level that is perhaps quite equivalent to morphology at a cellular level. It is expected therefore that genetic factors control not only the potentialities for the existence of various kinds of intracellular enzymes but also the intracellular orientation of the enzymes with respect to each other. Such factors should enter into a kinetic treatment of a reaction in a complex system in terms of concentrations of enzyme, substrates, etc., but it is not yet feasible to handle the problem in this manner. Obviously one of the very important problems of modern biochemistry is concerned with ascertaining more completely the functional significance and the mechanism of action of the organized enzyme systems in cells.

Intracellular organization was considered briefly in Chapter 2, where it was pointed out that certain definable particles and bodies in the cell, such as nuclei, mitochondria, microsomes, and plant plastids, possess enzymatic activity. All are bodies which can be removed from the cytoplasm by centrifugation, and all can be separated from each other in a more or less satisfactory manner. Hogeboom, Schneider, and Pallade (283) have provided one of the most elegant methods for the separation of the particulate materials from the cells of mammalian tissues. Briefly, their procedure is as follows: Tissue is removed immediately after the death of an animal, and, after having been cooled to below 2°C, it is homogenized in a 0.88 M sucrose solution. Centrifugation at $600 \times g$ for 10 minutes removes cell nuclei, unbroken cells, and other tissue residues. A second fraction containing large cell granules or mitochondria (0.5- to 2-μ diameter) is obtained by centrifugation at $24{,}000 \times g$ for 20 minutes. A third fraction containing ultramicroscopic particles or microsomes (60- to 150-mμ diameter) is obtained at $40{,}000 \times g$, while the fourth fraction, the final supernatant, contains the soluble enzymes. Actually there is good evidence that the mitochondria are enclosed in a fragile semipermeable membrane, and even though they tend to clump during isolation pro-

cedures they are not themselves simply coacervates. There are several questions concerned with the enzymatic activities of the complexes that are most significant in the field of genetics. That such complexes do exist in a wide variety of cells is hardly questionable. Since they do exist, how are the enzymes organized with respect to each other? Do different particles carry different functional systems? What is the mechanism by which the units of inheritance control the formation and maintenance of organized particles?

It will be recognized that the separation of cytoplasmic particles is on the basis of cytological and not chemical properties. Although the materials of each particulate fraction can be prepared so that they appear microscopically to be quite uniform, each may contain impurities of the others, and many different kinds of particles may be represented among the mitochondria and the microsomes.

Some approximate analytical information on these fractions is given in Table 14. The plus signs indicate, for the most part, a concentration

Table 14. Approximate Relative Compositions of Cellular Fractions

	Fraction			
Substance	Nuclei	Mitochondria	Microsomes	Supernatant
Desoxyribonucleic acid (DNA)	+++	−	−	−
Ribonucleic acid (RNA)	+	±	++	++
Lipoprotein	?	++	+	?
Cytochrome *c*	−	++	−	−
Cytochrome oxidase	+	++	−	−
Cytochrome reductase	−	−	++	−
Succinic acid dehydrogenase	−	++	−	−
Oxalacetic oxidase	−	++	−	−
Amino acid oxidase	−	++	−	−
Tyrosinase	−	++	−	−
Arginase	++	−	−	−
Uricase	++	−	−	−
Alkaline phosphatase	+++	−	−	−
Adenosinetriphosphatase	−	−	+++	−
Esterase	++	−	++	−
Catalase	+	−	−	++
Carboxylase		−	−	++
Lactic acid dehydrogenase	+	−	−	+
Amylase	−	+++	−	−
Krebs cycle system	−	+++	−	−
Glycolytic system	−	−	−	+++
Fatty acid oxidation system	−	+++	−	+++
Protein synthesis system	+	+	++	+

Table 15.* Distribution of Enzymes, Phosphorus-Containing Compounds, "Protein" Nitrogen, and Dry Material in Fractions of Rat Liver Homogenates (Average Values)

Tissue Fraction	Enzyme Activities			Micrograms Phosphorus per 100 Mg Fresh Tissue						Micrograms Protein N per 100 Mg Fresh Tissue	Milligrams Dry Material per 100 Mg Fresh Tissue
	Succinic Oxidase †	Cytochrome Oxidase †	ATP-ase ‡	DNA §	PNA §	Nucleic Acid	Acid Soluble	Lipid	Protein		
Original homogenate	383	1,012	865	22.6	65.2	95	125	155	40.2	1,970	31.9
Nuclear fraction	25.4	54.6	231	23.4	4.9	27.2	4.8	19	6.1	197	3.3
Mitochondria fraction	289	748	416		11.4	16	4.9	42.1	11.4	434	5.7
Unfractionated residue	45.5	147	257		47.8	61.5	115	95	19.7	1,250	23.3

* From Schneider and Potter (545).

† Activities are expressed as microliters of O_2 taken up in 10 minutes by the equivalent of 100 mg of fresh tissue.

‡ Activities are expressed as micrograms of phosphorus liberated in 15 minutes by the equivalent of 100 mg of fresh tissue.

§ Calculated from pentose determinations.

Table 16.* Oxalacetic Oxidase Activity of Rat Kidney Fractions

Nw = mitochondria; Mw = microsomes; S_1 = soluble fraction.

Tissue Fraction	Oxalacetic Oxidase Activity			
	3 Hours post Mortem		6.5 Hours post Mortem	
	Total Microliters O_2 per 10 minutes	Fraction of Homogenate, %	Total Microliters O_2 per 10 minutes	Fraction of Homogenate, %
Homogenate	226	(100)	187	(100)
Nw	18.0	8.0		
Mw	73.0	32.3	65.0	34.8
S_1	2.2	1.0		
Nw + Mw	141	62.4	115	61.5
Nw + S_1	13.0	5.8		
Mw + S_1	149	66.0	42	22.4
Nw + Mw + S_1	193	85.5		

* From Schneider and Potter (545).

of substance relative to the total and to the other fractions. The minus signs do not necessarily indicate a complete absence of the substance but only a relatively low concentration or activity. Thus, this information, most of which comes from studies on mammalian tissues, is presented only to show general trends of distributions. Some examples of quantitative data presented by Schneider (542) and by Schneider and Potter (545) are given in Tables 15 and 16. From Table 16 it should be noted that, although a fraction may be extremely low with respect to an enzyme activity, it may have a potent enhancing effect on the activity of another fraction. As shown in Table 14, in order to obtain an oxidation of glucose through glycolysis and the Krebs cycle it is necessary to recombine a supernatant with a mitochondrial fraction.

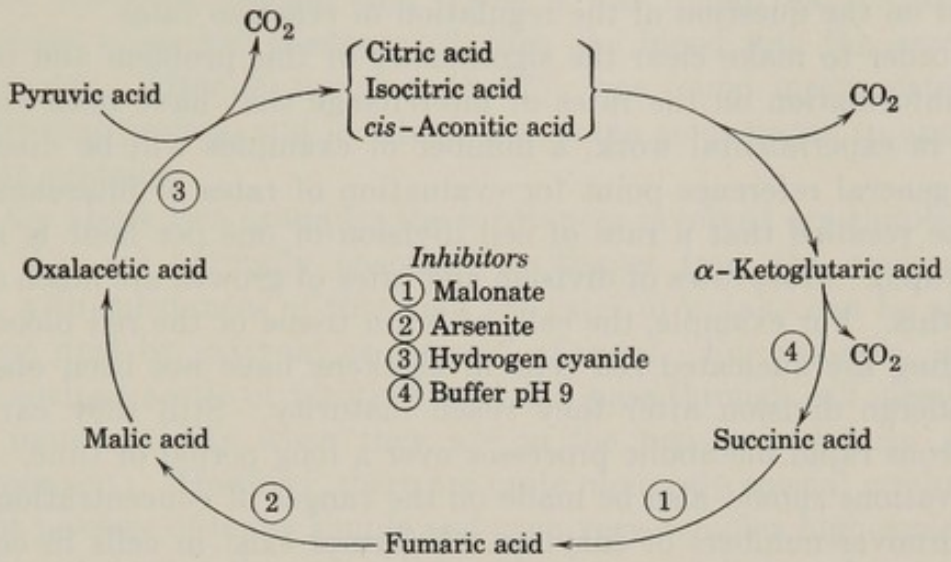
Now, to consider one of the complex systems in a little more detail, one of the most informative is that prepared and investigated by Green, Loomis, and Auerbach (225) and also utilized by Lehninger and coworkers (357). These workers prepared enzyme complexes from a number of rabbit tissues by centrifugation in salt solutions. According

to Schneider and Potter (545), this preparation, referred to by Green et al. as the cyclophorase system, contains a mixture of cellular particles. Green and coworkers were able to show that the particulate material has the capacity for the complete oxidation of pyruvic acid to CO_2 and water when it is allowed to act in the presence of oxygen or other oxidizing agent. Furthermore, it was demonstrated that this process occurs through the series of individual reactions described by the Krebs cycle (225). This was done by use of inhibitors of specific reactions of the cycle as indicated in Fig. 34. Other known necessary components of the cycle are also included in the figure. It has now been shown that similar preparations of cell particles as described above contain additional enzymes or systems such as oxaloacetic acid oxidase, ATP-ase, amylase, and systems that oxidize fatty acids. Lehninger (357) and Potter (112, 364, 493) and coworkers have made extensive investigations of the fatty acid oxidation system. All these activities appear to be associated principally with the mitochondria.

3. The Dynamic State

The opening paragraph of the first of a series of three lectures prepared for presentation in 1940 by Professor R. Schoenheimer (547) expresses in a simple straightforward fashion a basic principle which cannot receive too much emphasis in any consideration of the relations of genes to biochemical reactions. Schoenheimer stated: "The general title, *The Dynamic State of Body Constituents,* designates an attempt to consider . . . some results of modern biochemistry which suggest that all constituents of living matter, whether functional or structural, of simple or of complex constitutions, are in a steady state of rapid flux." That this concept was anticipated by several investigators, in particular Borsook and Keighley (59), was pointed out by Schoenheimer. As a result of these earlier ideas and as a result of the introduction of the use of isotopes as biochemical tracers, by Schoenheimer, his early associates, and his many followers, there began a great revision in biochemical thought. It became clear that chemical constituents taken in from the environment of an organism could no longer be considered as fuel, the combustion of which merely provides energy for the support of a relatively unchanging organic structure. Rather, it became evident that the molecular constituents of food undergo rapid interchange with like molecular constituents of cells, and the particular molecules that are oxidized to provide energy may come directly from the environment but may also come directly from apparently un-

changing structures of the organism. During periods of rapid growth the rates of direct oxidation and direct incorporation of food materials



ENZYMES AND OTHER SUBSTANCES KNOWN TO BE ASSOCIATED WITH THE CYCLOPHORASE SYSTEM

Enzymes	*Coenzymes and Other Substances*
Malic acid dehydrogenase	Coenzyme I (DPN)
Glutamic acid dehydrogenase	Coenzyme II (TPN)
Isocitric acid dehydrogenase	Diphosphothiamine
Succinic acid dehydrogenase	Adenosine triphosphate (ATP)
α-Ketoglutaric acid dehydrogenase	Flavin-adenine dinucleotide (FAD)
β-Hydroxybutyric acid dehydrogenase	Coenzyme A (pantothenic containing)
Adenosinetriphosphatase	
Cytochromes	

Additives Required

Substrate, any component of the cycle
Oxygen
Adenosine-5-phosphate (AMP)
Mg^{++}
$PO_4^{=}$

Fig. 34. Scheme for pyruvic acid oxidation by an enzyme complex (cyclophorase system) in the presence of oxygen. The upper part of the figure shows the principal intermediates and the inhibitors that have a specific action on the system. The lower part of the figure gives a list of components known to be part of the functional cyclophorase system.

into tissues may far outstrip rates of interchange. Nevertheless reversible and cyclic processes are always to be expected within the systems that provide energy and build tissue substance. It is this

ever-changing system of interrelated, reversible, and cyclic systems that is regulated by the units of heredity, and it is for this reason that the principal emphasis on the biochemical side of the problem has been placed on the question of the regulation of reaction rates.

In order to make clear the significance of this problem and to provide information on the rates of interchange that have been encountered in experimental work, a number of examples will be discussed. As a general reference point for evaluation of rates of interchange, it will be recalled that a rate of cell division of one per hour is indeed very rapid. Most rates of division and rates of growth are much slower than this. For example, the cells of brain tissue or the red blood cells including the nucleated red cells of chickens have not been observed to undergo division after they reach maturity. Still they carry on numerous rapid metabolic processes over a long period of time. Some observations should also be made on the ranges of concentrations and the turnover numbers of enzymes. Enzymes exist in cells in concentrations as high as 0.1%, and some possess turnover numbers as high as 5,000,000; i.e., 5×10^6 molecules of substrate are decomposed per minute by the action of one molecule of enzyme. Turnover numbers in the order of 100 are perhaps much more common. There is no basis for placing a definite lower limit for a significant enzyme concentration or turnover number. One molecule of enzyme with an extremely low turnover number may support an important metabolic function.

Membrane Permeability

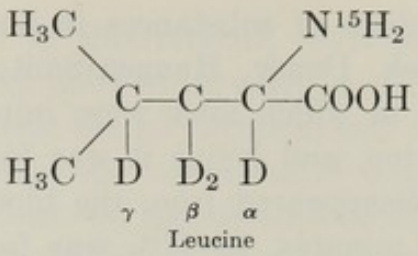
Hevesy (277) has provided an extensive discussion of data concerned with the use of isotopic tracers in measuring uptake of substances by cells as well as their use in measuring turnover or exchange rates after they enter cells. Substances normally absorbed are often taken up and metabolized with great rapidity. For example, as shown by Calvin and Benson (86), a 5-second exposure to light in the presence of $C^{14}O_2$ of a culture of the green algae *Chlorella* results in the production of measurable amounts of at least eight radioactive substances. The first substance detected is phosphoglyceric acid. With a 90-second exposure to light the number of labeled compounds at least doubles and sucrose is included in the products. Whether the actual uptake of CO_2 in this case is enzymatic or a process of diffusion cannot be stated. The uptake itself can be a seriously limiting factor even with small molecules or ions, and all the experiments on *in vivo* turnover rates are subject to this limitation. An excellent example showing the rate

of uptake and metabolism of substances in animals comes from investigations by Borsook, Deasy, Haagen-Smit, Keighley, and Lowy (64). In experiments in which mice were injected with C^{14}-labeled glycine, histidine, leucine, and lysine it was found that virtually all the amino acids had disappeared from the blood into the carcass in 10 minutes. After 30 minutes, 18–47% was found incorporated into the visceral protein, and in 1 hour the amino acids began to appear in plasma proteins.

In the above two examples the substances involved are simple molecules that are normally absorbed by tissues from the environment. Even with substances of this kind the rates of uptake can be affected a great deal by external or internal changes. For example, organic acids such as acetic or nicotinic acid will pass through cell membranes with more rapidity when they are in the non-ionized form (acidic environment). However, there are quite obviously special mechanisms for the passage of many simple and even very complex high-molecular-weight substances through cell membranes, and it is not possible to predict which substances will and which will not be taken up. As will be seen subsequently this phenomenon undoubtedly forms the basis for serious limitations to many methods for investigating the genetic control of metabolism.

The Metabolic Pool

As already mentioned, the early work with the tracer isotopes N^{15} (nitrogen^{15}) and D (deuterium) provided a conclusive demonstration of the rapid biochemical interchange of nitrogen and hydrogen atoms between various cellular substances. These results suggested the idea of a "metabolic pool"; that is, these atoms enter and leave numerous chemical combinations through some common intermediates such as NH_3 or H_2O. When this is true the atoms lose their identity as part of any given kind of molecule and undergo a continuous redistribution at rates dependent on the nature of their biochemical environments and on the nature of the chemical bonds that they form. The idea of the metabolic pool possesses no implications as to the biological site or mechanism of interchange of atoms; it only states that interchange of atoms does occur. An excellent example of the simultaneous use of N^{15} and D in a biological experiment comes from experiments by Sprinson and Rittenberg (588). The amino acid leucine was synthesized, using N^{15} and D in place of N^{14} and H to give a substance of the following composition.



Leucine

The labeled amino acid was then fed to rats, the animals were sacrificed, and the proteins were extracted from various tissues. These were hydrolyzed, and the leucine they contained was isolated in pure form. This leucine was found to contain D and N^{15}, a fact that demonstrates an interchange of dietary amino acid with tissue protein amino acid. The leucine was then degraded stepwise in order to determine the relative interchange of D in the α, β, and γ positions on the molecule. Results are given in Table 17. These data demonstrate the existence

Table 17.* Metabolic Changes in Deuterium–N^{15} Ratios of α-, β-, and γ-Deuterium Atoms of L-Leucine

	Leucine			Isolated/Fed	
Ratio D:N^{15}	Fed	From Carcass	From Internal Organs	Carcass	Internal Organs
Dα:N^{15}	1.46	0.38	1.53	0.26	1.05
Dβ:N^{15}	3.72	8.05	5.53	2.17	1.49
Dγ:N^{15}	1.15	2.45	1.83	2.13	1.59

* From Sprinson and Rittenberg (588).

of additional reactions besides those involved in the interchange of the whole amino acid with that of the tissue protein. First, the nitrogen atom exchanged independently of the relatively stable D-C bonds of the β and γ position. Secondly, the D, α atom exchanged with H atoms at an even higher rate than the nitrogen. Thus, from this relatively simple experiment the principles of the dynamic state and the idea of a metabolic pool can be understood clearly.

In the above example, the mechanism by which the interchange of the leucine molecule and its parts takes place is only partially known. Some kinds of interchange may take place when the molecule is uncombined in the tissue and some probably occur when the leucine is part of a protein molecule.

The same general principles indicated by the investigation outlined above have been applied in an enormous number of experiments using

many kinds of labeled atoms and molecules. For example, phosphorus, sulfur, and iron undergo rapid interchange. Among the carbon compounds it has become clear that forms of one- and two-carbon radicals exist that enter into a great variety of biochemical syntheses and degradations, and thus these too might be considered under the concept of a metabolic pool. It should be quite possible, eventually, to arrange biochemical substances, their organic radical constituents, and atom constituents into an almost continuous biological reactivity series, from those which undergo rapid interchange in many different ways to those which are completely inert in a biochemical environment.

Turnover of Material in Cell Nuclei

It is of particular interest for the present discussion to consider the interchange of materials of nuclei in relation to the turnover of other materials in the cells. It has already been indicated that desoxyribonucleic acid is confined to the nucleus in mammalian tissues. Thus an interchange in the components of DNA *in vivo* relative to an exchange of RNA which is found in other parts of the cell as well as in nuclei gives an indication of differences in rates of metabolic activities. Hammarsten and Hevesy (255) investigated the specific activities of DNA and RNA obtained from rat tissues 2 hours after subcutaneous injection of radioactive phosphate. Some of their results are summarized in Table 18. Results of a similar experiment in which the nucleic acids of a whole rat were similarly analyzed are given in Table 19. The results of both of these experiments demonstrate a very much lower turnover rate in the nucleic acid characteristics of the nucleus (DNA). This is especially evident in liver tissue, where little cell division is expected.

This phenomenon is further emphasized by some data obtained by Furst, Roll, and Brown (191). These investigators fed to rats adenine labeled in the 1 and 3 positions with N^{15} and then determined the turnover rates of the purines in DNA and RNA as indicated in Table 20. Again a very low rate of turnover in DNA was found. This is especially clear in the last two experiments shown in the table, where it is demonstrated that the labeled purine incorporated in DNA during rapid mitosis is largely retained over a long period of time, while the labeled purine in RNA disappears to a great extent during the same period. The authors of these experiments suggest that all of the incorporation of adenine by liver cell nuclei DNA occurs at cell division, and thus DNA has a fixed non-interchanging structure. However, since the ratio of interchange for adenine in liver cells is 73:1 for RNA to DNA

Table 18.* Rate of Renewal of Ribonucleic and Desoxyribonucleic Acids in Organs of Rats Two Hours after Administration of Labeled Phosphate

Organ	Nucleic Acid	Percentage Ratio of Specific Activity of Nucleic Acid P to That of P of Organ	Ratio of Specific Activity of Ribonucleic Acid P to That of Desoxyribonucleic Acid P
Liver	Ribose	3.45	33
	Desoxyribose	0.105	
Spleen	Ribose	6.6	3
	Desoxyribose	2.2	
Kidney	Ribose	6.1	2
	Desoxyribose	2.8	

* From Hammarsten and Hevesy (255).

Table 19.* Specific Activities of Nucleic Acid Phosphorus of Whole Rat, Liver, Spleen and Intestinal Mucosa

Sample	Specific Activity (Whole Rat Ribonucleic P = 100)		
	Ribonucleic	Desoxy-ribonucleic	Inorganic P
Total rat	100	60	
Liver	164	4.4	5,100
Spleen	292	63	2,850
Intestine	112	63	2,770

* From Hammarsten and Hevesy (255).

Table 20.* Incorporation of Adenine into Nucleic Acids

Experiment No.	Desoxyribonucleic Acids			Ribonucleic Acids		
	Total Purines †	Adenine †	Guanine †	Total Purines †	Adenine †	Guanine †
1. Mixed viscera, adenine fed 10 days		0.55	0.38		15.9	9.1
2. Non-growing liver, adenine fed 5 days	0.17	0.29			21.2	8.7
3. Regenerating liver, adenine fed 5 days	8.7	16.3	3.2	11.8	22.7	8.1
4. Regenerating liver, 26 days, no adenine after 5 days	6.5			1.8	2.7	1.7

* From Furst, Roll, and Brown (191).

† Calculated on the basis of 100% labeled adenine fed.

while the ratio for phosphorus (considered above) is 33:1, it may be that the structure is not completely inert.

In contrast to this behavior of DNA, experiments by Borsook and collaborators (63) indicate that the protein of the nucleus undergoes exchange of amino acids at a high rate. Some data are given in Table 21. It will be noted that, *in vivo*, the microsome fraction was the most

Table 21. Relative Incorporation Rates of Labeled Amino Acids into Liver Cell Fractions (Guinea Pig) *

Data are given as micromoles of amino acid per gram of protein per hour.

	Nucleus		Mitochondria		Microsomes		Supernatant	
Substance	*In Vitro*	*In Vivo*	*In Vitro*	*In Vivo*	*In Vitro*	*In Vivo*	*In Vitro*	*In Vivo*
Glycine	0.52	0.56	0.40	0.6	0.075	1.3	0.0	0.69
Histidine	0.32	1.5	0.15	1.2	1.5	3.1	3.7	1.2
Leucine	0.61	2.3	?	1.1	0.0	4.3	0.0	1.8
Lysine	4.1	1.3	3.2	1.6	0.9	2.9	3.2	1.6

* From Borsook et al. (63).

active but the nuclear fraction was also very active. When the incorporation experiments were carried out with the isolated fractions, the nuclear preparation was the most effective and it was a good deal more active than the most probable contaminating fraction which contains the mitochondria. Very little more information of this kind is available, and it is a matter of great importance to obtain much more extensive data concerning the metabolic turnover of all the chemical components of nuclei. Genes are persistent and reproducible biological entities, and the extremely low turnover of nuclear DNA suggests a stability to a biochemical environment that can be correlated with the stability of genes. Thus, the conclusion might be drawn that genes are made of desoxyribonucleic acid. On the other hand, it is well to note that biochemical stability can indeed be the result of inertness to the environment but it can also be the result of a balanced pattern of reactions with a high turnover of the substances that enter into the pattern. The stability of the dynamic process may be characteristic of genes just as it is of many cellular structures and biochemical reactions. It seems best to reserve judgment for the present as to whether genic substance is biochemically inert, labile, or both inert and labile.

4. Biochemical Reactions

The discussion so far in this chapter has been based on the contention that the units of heredity control, primarily, the relative rates of reactions. It has been pointed out that there are many possible ways in which rates can be affected even in relatively simple systems, and that even more variables must be considered when dealing with enzyme complexes. It is not known to what extent a reaction rate must be altered in order to produce an obvious phenotypic change in an organism. However, it is reasonable that a very small change in rate can result in an extreme morphological or biochemical alteration. For example, if substance A is an observed structural unit in a cell and it exists because it is produced at the same rate it is degraded, then a small reduction in the rate of its formation will result in the disappearance of substance A if the rate of degradation remains unchanged and if no other biochemical compensations exist.

In the discussion of reaction rates little attention has been given to the kinds of biochemical reactions that occur or to the mechanisms by which they take place. It is not within the scope of this book to discuss these problems in great detail, but some aspects of them have a direct bearing on the subject of reaction velocities and the genetic control of these velocities. Some of these points are discussed in the remainder of this chapter.

Energetics of Biochemical Reactions

When the chemical reactions of a cell reach a thermodynamic equilibrium the cell is dead, and it is well decomposed. Cells exist in life at the expense of the energy supplied by the environment and by virtue of their capacities to extract and store, for a future time, energy and materials from the environment. The cell wastes rather than conserves energy, and its apparent defiance of thermodynamic laws by its extraordinary degree of organization is an artifact arising from the dynamic state of its existence.

As already noted, many investigators in the field prefer to consider a living system as existing in a steady state with respect to its biochemical processes. This of course can be true literally only over a short period of time since cell divisions, adaptation to environmental changes, and aging certainly introduce elements of unsteadiness in cellular processes. In actual fact it must be considered that the rates

of reactions will not be precisely the same over a finite interval of time.

In the photosynthetic organisms, the energy required for maintenance of the system is derived from light energy, whereas in other organisms it is obtained from the energy of the chemical bonds of the substances taken up from the environment.

In both kinds of cells the reactions by which carbohydrate is formed and broken down are of fundamental importance since they provide

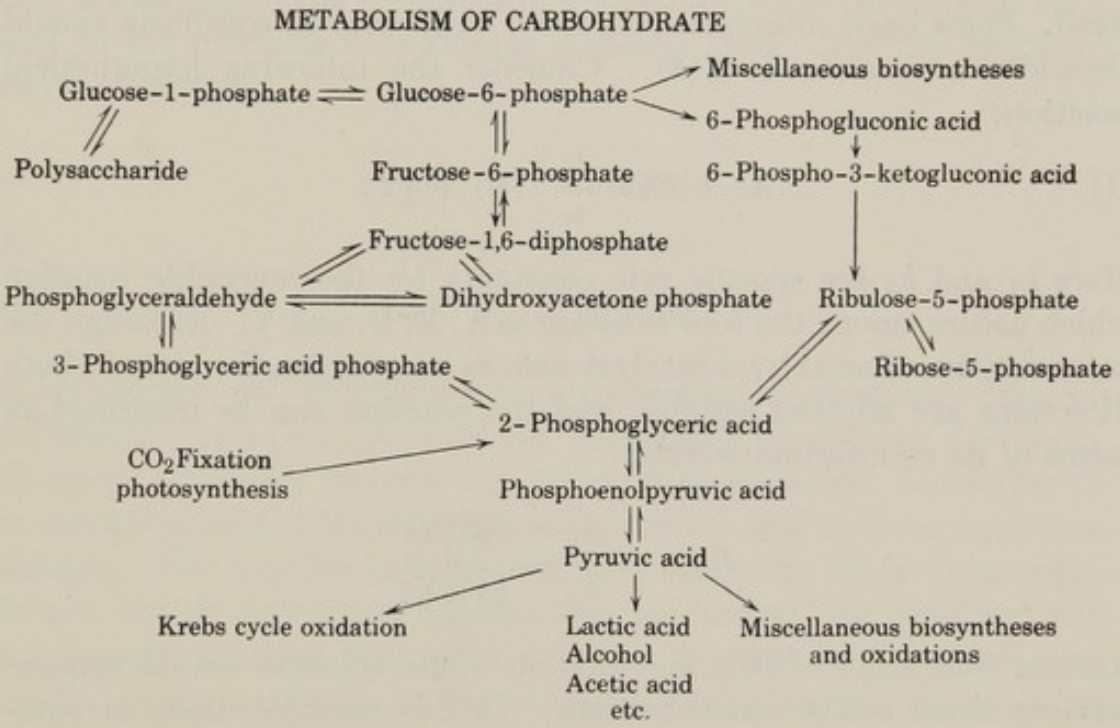


Fig. 35. Scheme showing some pathways of metabolism of carbohydrate.

the pathways by which much of the light or chemical bond energy can be conserved and redistributed among a great variety of new chemical bonds. A general pattern of carbohydrate metabolism is presented in Fig. 35. This scheme is by no means complete, and further details can be found in numerous discussions (139, 345, 363). The pattern as given is adequate to provide a general reference for the discussions that follow. Particular note should be made of the fact that several of these reactions involve the formation of phosphate esters or anhydrides. As will be discussed later such substances are extremely important in the transfer of energy in metabolic processes. On the other hand it should be kept in mind that the organic derivatives of phosphoric acid are not the only substances that are used in energy transfer.

Coupled Reactions

The biochemical reactions of living cells occur in consecutive series with a very large number of divergences and convergences of the metabolic pathways. Although some reversible reactions may reach equilibrium, in general there is a more or less steady flow of materials from those taken in from the environment to those that are excreted back into the environment. Nevertheless, the potential equilibrium state of each reaction in any series is important in determining the course of the reaction pathway as well as the composition of the tissue itself. Some basic principles concerned with reaction equilibria should therefore be examined (318). Consider the following hypothetical reaction:

$$(1) \qquad (A) + (X) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (B) + (Y)$$

Here k_1 and k_2 are specific rate constants for the reversible reaction which occurs among the four substances A, X, B, and Y. Although the rates may be affected by a catalyst such as an enzyme, the rates in both directions are affected equally, and the reaction can be described in terms of its equilibrium constant:

$$K_{eq\ 1} = \frac{k_1}{k_2} = \frac{(B)(Y)}{(A)(X)}$$

Assume that $K_{eq\ 1} = 0.01$, and that in dilute solution actual concentrations equal active concentrations. In this example, then, at equilibrium: (A) (X) = 100 (B) (Y) and a high concentration of A or X is required to force the production of a much smaller amount of B or Y. This necessity for a high concentration of one or the other or both reactants can be avoided by addition of a second reaction to the system as shown below:

$$(2) \qquad (B) \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (C) + (D)$$

Here $K_{eq\ 2} = \frac{k_3}{k_4} = \frac{(C)(D)}{(B)}$, and it is assumed, for the present purpose, to have a value $K_{eq\ 2}$, = 1000.

These reactions, 1 and 2, can now take place in series through the common substance (B). Since the equilibrium in (2) is very much in favor of formation of the products C and D the concentration of B in the system can be reduced to a very low value and reaction 1 can pro-

ceed without the necessity of having a high concentration of A or X. The over-all reaction will then be:

(3) $$(A) + (X) \rightleftharpoons (Y) + (C) + (D)$$

where $K_{eq\ 3} = (K_{eq\ 1})(K_{eq\ 2}) = 10$.

In quantitative terms and in units of energy it can be shown that the standard free-energy change during a reaction $\Delta F^\circ = RT \ln K_{eq}$. This value represents the required change in free energy of the system for a conversion of the reactants in a standard state (in a molal solution) to the products in a similar standard state. For the reactions above, at 25°C:

(1) $$\Delta F^\circ = +2{,}740 \text{ cal.}$$

(2) $$\Delta F^\circ = -4{,}110 \text{ cal.}$$

(3) $$\Delta F^\circ = -1{,}370 \text{ cal.}$$

By convention, ΔF° is negative for a spontaneous (exergonic) reaction and positive for an energy requiring (endergonic) reaction. Obviously any single reversible reaction can go in either direction, depending on the concentrations of the reactants and products and on the equilibrium constant. No reaction can proceed in a direction away from equilibrium, but an endergonic reaction can be "driven" by coupling with an exergonic reaction through a common reactant and through consecutive steps, as shown in the example given above. It should be noted that the reaction velocities in the single or consecutive reactions are not dependent on the equilibrium constants but are functions of the properties of the individual catalysts of the system. That is, the catalysts determine the actual course and velocity of a reaction while the over-all free-energy change indicates the potentiality for the occurrence of a reaction. As discussed later (p. 143), the mechanism of an enzyme-catalyzed reaction can be very complex, and coupling increases this complexity.

Coupling is an essential feature of biochemical processes. It makes possible the flow of materials through long series of reactions without the necessity for the accumulation of excessive amounts of intermediates just prior to a reaction in which the equilibrium is unfavorable for the direction of flow of substances. In any given series of biochemical reactions there may be only one that requires coupling or there may be many, either immediately following one another or

placed at intervals along the chain. A consideration of these facts about the energetics of chemical reactions yields some important conclusions pertaining to the effects of genetic changes on metabolism. First, with regard to any reaction that normally operates in the vicinity of equilibrium, it is obvious that a genetic change which alters the concentration of one of the reacting substances can change the direction of spontaneous flow of materials. The result might be the accumulation of an intermediate, a reversal of the direction of flow of materials, and perhaps a loss in adequate capacity for synthesis of some essential substance. A second point of significance arising from a consideration of coupled reactions is that a genetic change which results in the loss of the exergonic step of a coupled system results also in the loss of one or more dependent endergonic steps. If the series of dependent endergonic steps is long, a number of different mutations which block the series in different ways and at different points can be expected to yield essentially the same phenotype.

In living cells there exists a wide variety of substances which, through their oxidation or reduction, hydrolysis, or other chemical changes, can yield energy for coupling. These include hydrogen carriers, peptide bonds, various kinds of esters, hemiacetals, and many other kinds of compounds. It is now well established that the esters, anhydrides, and amides of phosphoric acid are particularly important in the transfer of energy in biological systems. Hydrolysis of these substances serves as the driving reaction for coupling with a great many endergonic systems. For example:

Glucose + Fructose + Adenosinetriphosphate →

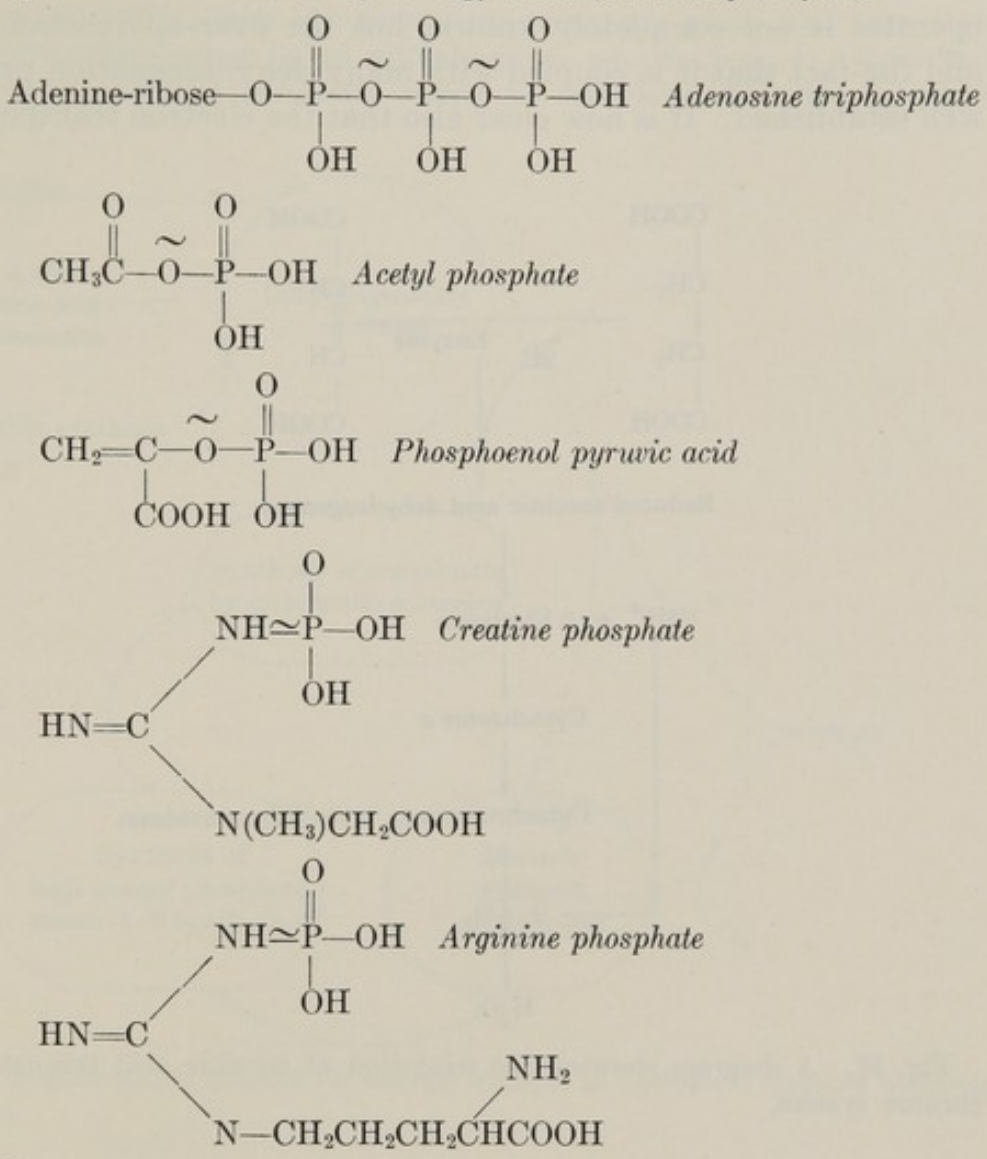
Sucrose + Adenosinediphosphate + H_3PO_4

Some of the biologically important derivatives of phosphoric acid are listed in Fig. 36.

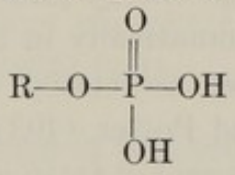
Synthesis of High-Energy Phosphate Bonds

The utilization of the energy of chemical bonds for the synthesis of high-energy phosphate bonds (Fig. 36) occurs through oxidation by various and often complex mechanisms. For example, the oxidation of pyruvic acid through the Krebs cycle (see Fig. 34, p. 129) does not use oxygen directly but it is coupled with the cytochrome system for the transfer of electrons to oxygen as indicated in Fig. 37. Here only the step of dehydrogenation of succinic acid is shown. The cytochromes are iron porphyrin-containing proteins which transfer electrons by

Group 1. High-energy anhydrides and amides 11 to 14,000 cal per mole. The symbol ~ indicates a high-energy bond (toward hydrolysis).



Group 2. Low-energy esters. 2,000–3,000 cal per mole.



R represents a variety of substances including sugars, the hydroxyamino acids, and phospholipid constituents such as ethanolamine and choline. The sugar phosphates include glucose-6-phosphate, fructose-1,6-diphosphate, the ribose and desoxyribose esters in nucleic acids and coenzymes, and the phosphate esters of the three carbon intermediates in glycolysis.

Fig. 36. Some biochemically important phosphate esters, anhydrides, and amides.

oxidation and reduction of the iron. The exact nature of all the components and the mechanism by which this electron transport system operates is not completely known, but the over-all reaction is clear, and the fact that it is coupled with many dehydrogenation processes is well established. It is now clear also that the electron transport system

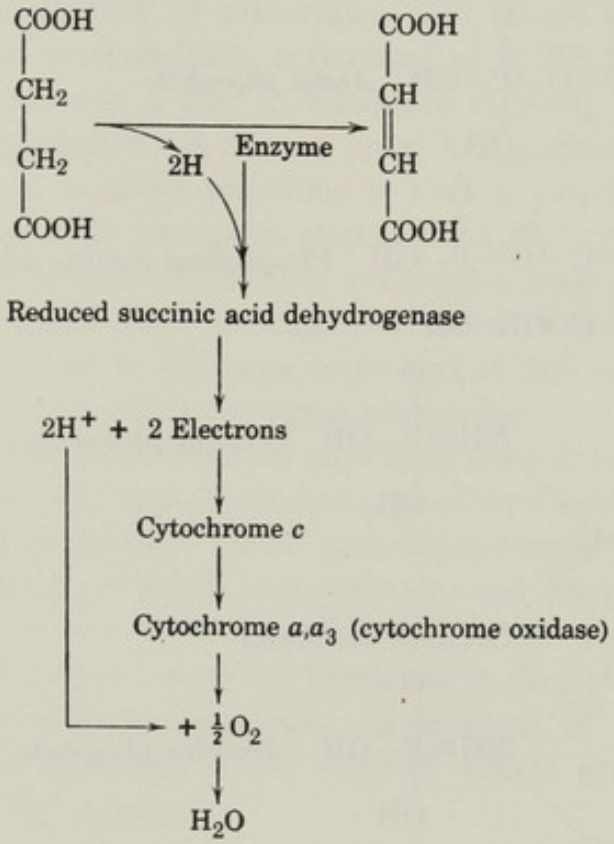


Fig. 37. A diagram showing the oxidation of succinic acid through the cytochrome system.

is coupled in turn with reactions that form high-energy phosphate ester bonds. These relations are indicated diagrammatically in Fig. 38.

Numerous investigators, in particular those associated with Green (225), Lehninger (357), Schneider (543), and Potter (493), have obtained evidence that the oxidation of pyruvate and of fatty acids occurs in the particulate complex enzyme systems that have already been discussed. Cross et al. (119) consider that the whole process functions in a very closely knit system in which the enzymes have collective properties not characteristic of the individual separable enzymes.

Each of the oxidative enzymes of the cyclophorase complex contains a coenzyme which is firmly bound to the protein moiety. These coenzymes are the instruments by which inorganic phosphate becomes esterified. As the coenzyme

undergoes reduction, simultaneously inorganic phosphate becomes esterified and a coenzyme pyrophosphate is formed. Each of the primary coenzymes reacts with oxygen through a series of intermediary coenzymes which also have their cycles of oxidation-reduction linked with phosphate esterification. It follows that esterification of inorganic phosphate cannot be dissociated from the oxidative process.

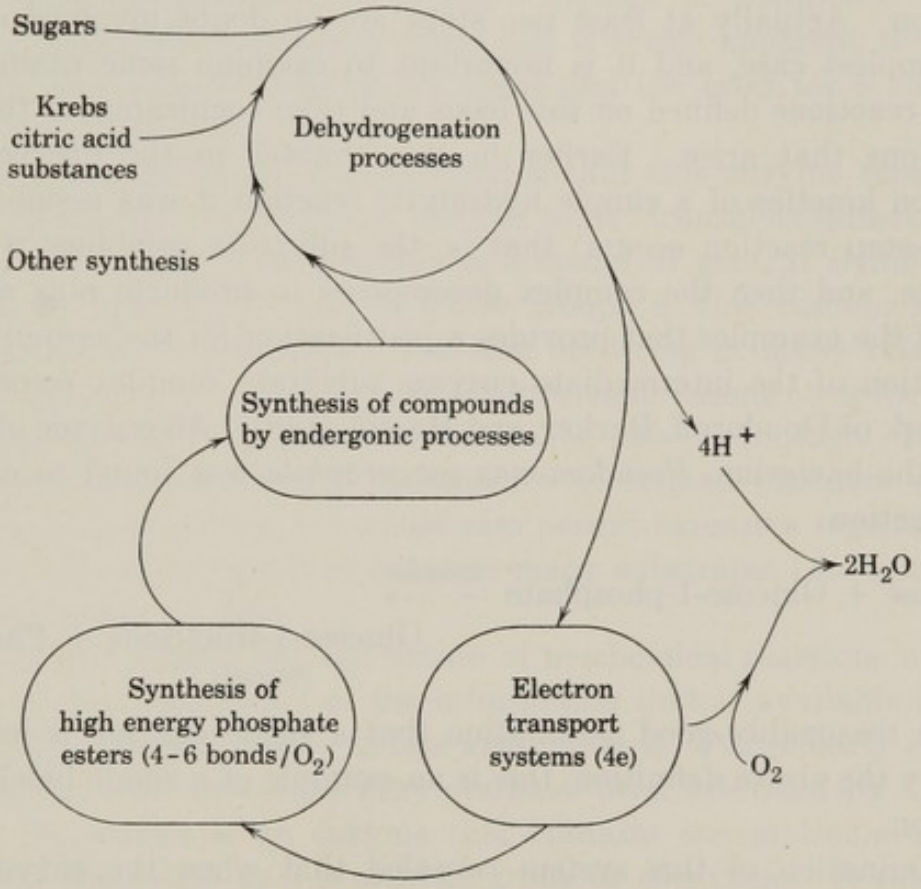


Fig. 38. A generalized scheme for energy transfer in biological systems utilizing free oxygen.

The above quotation summarizes a proposed mechanism for synthesis of high-energy phosphate bonds that is consistent with a large number of experimental facts.

On the Nature of Biochemical Reactions

It is by no means a simple matter to produce a rigorous definition of a single biochemical reaction. And yet this is an important question when it is desired to know the mechanism of genetic control of biochemical reactions. The difficulties are not confined to descriptions of enzyme-catalyzed conversions since innumerable uncatalyzed chemical reactions are complex in nature, and, even though they may be stoichiometric over all, they can have consecutive, simultaneous, or chain-reaction mechanisms.

Perhaps then it will be more satisfactory to define a single biochemical reaction in terms of the catalyst that participates in the change. Thus, if substance A is converted to substance B under the influence of an enzyme preparation that contains only one molecular species it is reasonable to call the reaction $A \xrightarrow{E} B$ a single biochemical reaction. Actually at least two steps are no doubt involved even in the simplest case, and it is important to examine some examples of single reactions defined on this basis and take cognizance of the complications that arise. Earlier in this chapter in the discussion of reaction kinetics of a simple hydrolytic reaction it was assumed that a two-step reaction occurs; that is, the substrate combines with the enzyme, and then the complex decomposes to products plus enzyme. One of the examples that provides a justification for the assumption of formation of the intermediate enzyme substrate complex comes from the work of Doudoroff, Barker, and Hassid (148). An enzyme obtained from the bacterium *Pseudomonas saccarophila* was found to catalyze the reaction:

$$\text{Fructose} + \text{Glucose-1-phosphate} \xrightarrow{\text{Enzyme}} \underset{\text{(Sucrose)}}{\text{Glucose-1-fructoside}} + \text{Phosphate}$$

It is a reasonably good assumption that a single enzyme is involved, and, by the above definition, this is an example of a single biochemical reaction.

Examination of this system revealed that when the enzyme was mixed with glucose-1-phosphate in the presence of inorganic radioactive phosphate, the glucose-1-phosphate rapidly became labeled. Thus it became evident that the first reaction is:

$$1 \quad \text{Glucose-1-phosphate} + \text{E} \rightarrow \text{Glucose-E} + \text{Phosphate}$$

The remainder of the reaction can be written:

$$2 \quad \text{Glucose-E} + \text{Fructose} \rightarrow \text{(Glucose-E-fructose)}$$

$$3 \quad \text{(Glucose-E-fructose)} \rightarrow \text{E} + \text{Sucrose}$$

It was found that several other sugars can be substituted for fructose in the reaction and that it is not necessary to start with the phosphate ester to effect the interchange. A disaccharide such as sucrose will do as well. The essential part of the reaction is therefore the combination of the glucose with the enzyme in such a fashion as to retain the neces-

sary bond energy to effect a transfer of the glucose to various acceptors. It should be noted that, in this reaction as written, there are actually at least two and perhaps three reversible steps, each of which can be more or less independently influenced (in rate) by the surrounding chemical or physical environment. It was shown, for example, that glucose is a potent inhibitor in the exchange of phosphate in the glucose-1-phosphate-enzyme system, and it seems apparent that this is due to competition between the sugar and the ester for a reaction site on the enzyme (step 1 above).

Doudoroff et al. (148) have suggested that this enzyme represents a class of catalysts that may be referred to as "transglycosidases," and it should be pointed out that this is a model of general significance. The transfer or interchange of whole groups in this fashion is now known to be a widespread phenomenon occurring in many relatively simple reactions and probably in interconversions among complex lipids, nucleic acids, and proteins as well as among polysaccharides. It is an obvious way of obtaining a multiplicity of related products with a minimum loss of energy. It would also permit extensive regulation of reaction rates by competition between many substrates (multiple substrate inhibition, p. 121).

Still further insight into the nature of biochemical reactions may be gained by an examination of the information that is available on the mechanisms of action of peroxidase and catalase. Particularly elegant experiments with crystalline enzymes have been described by Chance (103). Peroxidase is an enzyme that contains one molecule of iron porphyrin (hematin) as a prosthetic group or coenzyme. It catalyzes the general type reaction:

$$\mathrm{R{-}O{-}O{-}H + AH_2} \xrightarrow[\substack{\text{(Enzyme)}\\ \text{Per{-}OH}}]{} \mathrm{ROH + A + H_2O}$$

where A can be a phenol, an aromatic amine, or some of a few other kinds of substances, and ROOH can be H_2O_2 or a substance such as methyl or ethyl hydrogen peroxide. Chance has demonstrated, by spectrophotometric means, the existence of two enzyme substrate intermediate complexes in the reaction, and he has studied the kinetic aspects of the several steps involved in the over-all reaction. The steps were formulated as follows:

(1) $$\underset{\text{(Enzyme)}}{\mathrm{Per{-}OH}} + \mathrm{ROOH} \rightleftharpoons \underset{\text{(Complex I)}}{\mathrm{Per{-}OOR}} + \mathrm{H_2O}$$

(2) $$\underset{\text{(Complex I)}}{\mathrm{Per{-}OOR}} \rightleftharpoons \underset{\text{(Complex II)}}{\mathrm{Per{-}OOR}}$$

$$(3)\qquad \underset{\text{(Complex II)}}{\text{Per—OOR}} + AH_2 \rightharpoonup \underset{\text{(Enzyme)}}{\text{Per—OH}} + ROH + A$$

It seems possible that reaction 3 should be broken down into two steps with a third intermediate complex Per—OOR—AH_2, but these two steps may be simultaneous.

The action of catalase (102) is similar in some respects to that of peroxidase, but it demonstrates some additional points of interest. This enzyme contains four hematin molecules per molecule of protein, and it can act as a peroxidase in the oxidation of alcohols or as a catalyst for the decomposition of hydrogen peroxide. When the enzyme acts as a peroxidase the over-all reaction can be formulated

$$ROOH + R'CH_2OH \xrightarrow[Cat(OH)_4]{\text{(Enzyme)}} ROH + R'CHO + H_2O$$

Stepwise the reaction has been described by experimental data, also obtained by spectrophotometric methods, as follows:

$$(1)\qquad \underset{\text{(Enzyme)}}{Cat(OH)_4} + 4ROOH \rightleftharpoons \overset{\text{(Complex I)}}{Cat(—OOR)_4} + 4H_2O$$

$$\Downarrow\Uparrow$$

$$\underset{\text{(Complex II)}}{Cat(—OOR)_4}$$

$$(2)\qquad \underset{\text{(Complex I)}}{Cat(—OOR)_4} + 4RCH_2OH \rightarrow \underset{\text{(Enzyme)}}{Cat(OH)_4} + 4ROH + 4R'CHO$$

In a somewhat similar fashion the decomposition of hydrogen peroxide,

$$2H_2O_2 \xrightarrow[Cat(OH)_4]{\text{(Enzyme)}} 2H_2O + O_2$$

was described by Chance as follows:

$$(1)\qquad \underset{\text{(Enzyme)}}{Cat(OH)_4} + H_2O_2 \rightleftharpoons \overset{\text{(Complex I)}}{Cat(OH)_3(—OOH)}$$

$$\Uparrow\Downarrow$$

$$\underset{\text{(Complex II)}}{Cat(—OH)_3(—OOH)}$$

$$(2)\qquad \underset{\text{(Complex I)}}{Cat(OH)_3(OOH)} + H_2O_2 \rightarrow \underset{\text{(Enzyme)}}{Cat(OH)_4} + H_2O + O_2$$

In these two examples, as with peroxidase, at least three steps have been shown to occur. However, in contrast to the peroxidase-catalyzed steps, these are not consecutive, since complex II does not enter directly into the final reaction. It will be noted also that H_2O_2 does

not combine with the enzyme to the same extent as do organic peroxides. In addition, hydrogen peroxide enters into both steps shown for its decomposition, so that the kinetics of the over-all reaction do not follow the formulation of Michaelis and Menten or of Straus and Goldstein.

It should be remarked that the above examples are among the best that demonstrate the formation of enzyme substrate complexes. It is not necessarily true, however, that all enzyme-catalyzed reactions do occur by formation of intermediate substances that have an existence for a finite period of time. This poses an unsolved problem as does the question of the mechanism and the steps involved in reactions catalyzed by enzyme complexes. With the latter kind of system there are obvious difficulties in defining a reaction on the biological basis that has been in use here, i.e., the over-all reaction catalyzed by a single enzyme. Still, it seems to be a more useful concept for the present purpose than would be the use of a single chemical step as a single biochemical reaction. The principal purpose, however, in the discussion of the nature of biochemical reactions has been to indicate in more detail some additional possibilities with respect to the ways in which reaction rates can be influenced. In connection then with the problem of the mechanism of genetic control of metabolism it will be convenient to consider that the rate of a single biochemical reaction, whether the reaction occurs under the influence of an enzyme of a single molecular species or under the influence of an enzyme complex with unique collective properties, can be very greatly influenced in two general ways:

1. The enzyme itself can be altered in quality or quantity.
2. The different chemical steps of an over-all reaction can be influenced individually by changes in the biochemical or physical environment.

CHAPTER 7

Mutations and the Agents of Metabolic Control

In 1945, Beadle (26) summarized an accumulation of genetic and related biochemical information and set forth a general hypothesis relating inheritance to biochemical processes. The statement of the hypothesis is as follows:

> As a framework on which to arrange conveniently the varied observations and inferences bearing on the nature of genes and their action it is desirable to have set down a definite summary hypothesis. The presentation of such a hypothesis is undertaken with the realization that alternatives are not only possible but in at least certain respects equally plausible.
>
> In order to exist as such, genes obviously must be capable of inducing the formation of exact copies of themselves. . . . In addition to catalyzing formation of more units like themselves, genes in general have heterocatalytic properties, that is they catalyze the formation of other substances. . . . In determining the specific chemical and perhaps physical configuration of protein molecules, genes directly determine enzyme specificities and thereby control in a primary way enzymatic synthesis and other chemical reactions in the organism. . . . Each of these thousands of gene types has, in general, a unique specificity. This means that a given enzyme will usually have its final specificity set by one and only one gene.
>
> If such a mutational change abolishes the autocatalytic property of the gene, the gene is irreversibly lost. On the other hand, if it loses its heterocatalytic power it remains a gene but so far as its effect on the organism of which it is part is concerned it becomes inactive. . . . Other types of gene mutations presumed to be possible are those in which the heterocatalytic property is impaired but not destroyed (hypomorphs), those in which the effectiveness of heterocatalysis is increased (hypermorphs), and finally those in which there is a change in one step from one heterocatalytic specificity to another (neomorphs).

More recently Beadle (27) revised the hypothesis in one of its essential parts with the suggestion that one gene has one primary function, thus including the formation of biochemical units other than enzymes.

This more generalized idea is very similar to that presented earlier by Grüneberg (236).

That the units of heredity function by exerting control over metabolism cannot be doubted. What remains to be discovered is the mechanism by which genes exert their control, and what biochemical agents are the substances most directly affected. From the biochemical standpoint metabolic processes are made up of a great variety of biochemical reactions, and, though the rates of these individual reactions can be influenced by many factors (see p. 164), it is clear that the proteins that have chemically specific binding capacities occupy a central position as potential primary agents of metabolic control. The biochemical catalysts, the enzymes, are of course included in this group of substances. In essence the hypothesis of Beadle quoted above suggests that: (1) a gene has an autocatalytic function in its own reproduction; and (2) a gene has a single heterocatalytic function, and, in relation to enzymes, it determines enzyme specificities directly. This means that each gene is unique and each enzyme or other molecule with a biologically specific function must obtain its specificity from a corresponding gene. It is presumed that mutation can give rise to loss or change in autocatalytic and heterocatalytic functions.

This hypothesis applied "as a framework on which to arrange conveniently the varied observations and inferences bearing on the nature of genes and their action" (26) directs attention to the central problem concerned with how inheritance is related to metabolic processes, and it suggests a concrete approach for investigating this relationship. The ideas and arguments that have arisen (27, 293) from attempts to prove or disprove the hypothesis have been numerous, but they have not provided an adequate answer one way or the other. Some of the reasons for this should be made clear by the following questions:

1. Exactly what is a single gene in physical and chemical terms? Experimentally, it is a unit defined by crossing over, but is it justifiable to equate this to a unit of mutation or a unit of function? (See Chapter 9.)

2. Do we know exactly what is meant by the term specificity? Can two macromolecules that are not identical have the same biochemical specificity?

3. Are the enzymes and other specifically functional substances that we have been able to isolate biochemically unreactive and produced only under the direct influence of genes? Are these substances as we know them in the isolated state identical in structure and action to the same materials *in vivo?*

4. Is the expression of a mutant phenotype as a deficiency or change in the properties of a substance, such as an enzyme, an adequate reason to consider the substance produced in the normal state to be a direct product of gene action?

5. Is it a necessary postulate that all genes function by the same mechanism?

A number of these questions have been anticipated and discussed earlier in this book, and others will receive consideration in the pages that follow. Many of the difficulties involved in this problem of providing a clear picture of heredity, on a biochemical basis, must already be obvious.

Without doubt, a phase of biochemistry about which it is necessary to have much more information in order to obtain answers to the many questions that have been raised is that concerned with the nature, the biological formation, and the fate of the agents of metabolic control, that is, whatever agents carry out the heterocatalytic function described above. Although small molecules can be included here, the present usage pertains particularly to the large molecules that go to make up the enzymes and enzyme complexes or the non-catalytic units that affect reaction rates. As indicated above, such units may be direct products of gene action, but it is also probable that many of those with which we work in the laboratory arise by the interaction of these substances among themselves without the direct intervention of genes. It was noted, for example, that a preparation of cell-free soluble enzymes is capable of incorporating isotopically labeled amino acids into protein (Table 21, Chapter 6). From this, Borsook and Deasy (60) drew the highly significant conclusion that the incorporation of labeled amino acids into proteins in the adult cell does not necessarily depend on the direct participation of the nucleus. This important proposition can be supported in other less direct ways, and it is probably applicable also to macromolecules other than proteins such as the nucleic acids and polysaccharides. Some of the important experimental facts pertaining to non-nuclear conversions of intracellular catalysts are considered in the following, necessarily limited, discussion of macromolecules.

1. Macromolecules

The molecules of biological importance have a very wide range of molecular weights, extending from 2.016 for H_2 to several millions in some of the more complex proteins, polysaccharides and nucleic acids. Generally speaking, the same kinds of chemical bonds are found in

small and large compounds, and, indeed, most of the high-molecular-weight substances are made up of well-known units of smaller substances linked together in a chain or network. It is not possible to assign a lower limit of molecular weight to the substances referred to loosely as macromolecules. The ribonucleic acids with molecular weights as low as a few thousand are usually included in the category. The enzymes that have been studied are all macromolecules. Such substances, by virtue of their size, the flexibility of long chains, and the presence of many functional groups, can assume a great variety of different orientations in space. Then, because of intermolecular attractions, these large molecules can assume the specific spatial configurations that are apparently essential to their biological functions.

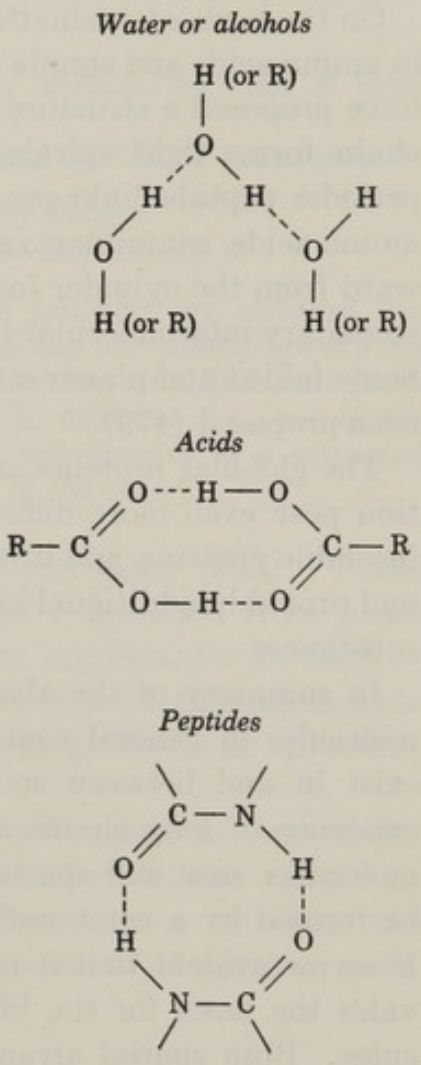


Fig. 39. Some illustrations of hydrogen bonding. R = an organic radical.

It is clear that one of the most important forces that act in the production and maintenance of such specific spatial configurations, as well as in determining other properties of biologically significant macromolecules, is the hydrogen bond or hydrogen bridge. This type of chemical bond is by no means confined to macromolecules. It occurs between hydrogen and all of the more electronegative elements. For example, H_2O exists in a highly associated state at ordinary temperatures as a result of hydrogen bonding. See Pauling (478) for discussion. Hydrogen thus forms quite stable bridges between a great variety of simple and complex molecules. Those of particular importance in biochemistry are: —O—H---O= as in water, peroxide, alcohols, acids, etc., and —N—H---O= as in glycine, urea, and peptides (Fig. 39). These secondary bonds are essentially ionic in character, and the bond energies are about 5 kcal/mole as compared to 110 and 83 for O—H and N—H bonds respectively.

In addition to hydrogen bonding, macromolecules form other intermolecular linkages, including those such as disulfides, salts between

functional groups, and coordination complexes between functional groups and metal ions. The contribution to total structure of a large molecule made by the various possible kinds of interaction is, of course, dependent on the relative positions of interacting groups as well as on the physical and chemical environment.

On the basis of evaluations of interatomic distances and bond angles in amino acids and simple peptides, Pauling, Corey, and Branson (480) have proposed a structure for some protein fibers in which the peptide chain forms tight spirals maintained by hydrogen bonding between periodic peptide linkages. In such a structure the R groups of the amino acids, containing various functional groups, extend radially outward from the cylinder formed by the helical chain and the significant secondary intermolecular bond is =N—H----O=, the hydrogen bond. Some folded and planar structures for other kinds of proteins have also been proposed (479).

The globular proteins and conjugated proteins and all those in solution pose even more difficult structural problems than do the fibrous insoluble proteins, and it is possible to state at present only that similar and probably additional kinds of intermolecular bonding occur in these substances.

In summary of the above discussion, it can be stated that macromolecules in general contain the same kinds of chemical bonds that exist in and between small molecules. However, by virtue of the existence of long chains and interacting functional groups in macromolecules, new and spatially specific networks of ring structures can be formed by a combination of inter- and intramolecular interaction. It seems evident that it is the existence of these large rings that provides the basis for the biologically specific properties of macromolecules. Both spatial arrangements and the accompanying distribution of electrostatic charges contribute to the phenomenon of biological specificity.

Variations in Specific Macromolecules

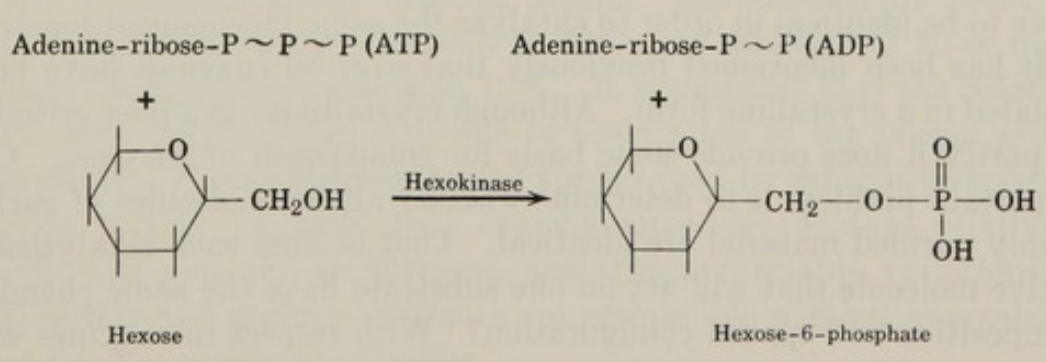
It is a well-known fact that enzymes obtained from different species of organisms are not necessarily alike even though they may catalyze the same reactions. They may differ not only in size and in the properties of their protein portions (apoenzyme) but also in the properties of the most specific functional portions that have to do with their combination with substrates and their specificities with respect to substrates. Some examples of such differences are given in Table 22.

Table 22. Some Species Differences in Crystalline Enzymes

Enzyme	Origin	Activity Difference
Glutamic acid dehydrogenase	Yeast or *E. coli*	Requires TPN as hydrogen acceptor
	Higher plant	Requires DPN as hydrogen acceptor
	Animal	Requires TPN or DPN as hydrogen acceptor
Alcohol dehydrogenase	Yeast	Completely inhibited by 0.001 *M* iodoacetate
	Animal	Not inhibited by 0.01 *M* iodoacetate
Glucose dehydrogenase	*E. coli*	Inhibited by toluene
	Animal liver	Not inhibited by toluene
Pepsin	Bovine Swine	Crystalline products are identical immunologically and solubilities are the same. However, solubilities are independent in mixtures (i.e., one of the proteins will dissolve in a saturated solution of the other).

Another can be found in the report of Tint and Reiss (653). These investigators demonstrated that cytochromes *c* obtained from heart muscle of beef, horse, pig, and chicken differ significantly in electrophoretic mobilities. Similar criteria have been used to demonstrate differences between species with respect to hemoglobins, egg albumins, and hemocyanins.

An investigation of Slein, Cori, and Cori (567) provides some interesting information on the hexokinases of different animal tissues as compared to the same enzyme in yeast. This enzyme catalyzes the transfer of phosphate from ATP to the six position of glucose, mannose, or fructose.



The crystalline enzyme was obtained from yeast, whereas a partly purified but adequately resolved preparation from sheep brain was utilized in the experiments. Partially purified enzymes were also ob-

tained from rabbit muscle, rat muscle, and rat liver. Using these preparations the following pertinent facts were ascertained:

1. All the preparations require Mg^{++} for hexokinase activity.

2. The yeast and brain enzymes cause phosphorylation of the three sugars with a definite ratio of rates in the order fructose > glucose > mannose. Extensive fractionation of the brain preparation did not change this ratio.

3. That the same reactive site is involved in each enzyme (yeast and the brain) was shown by inhibition studies with the three sugars. Glucose and mannose gave a mutual inhibition, whereas fructose was only slightly inhibitory to phosphorylation of the other sugars. (Common substrate inhibitions are discussed on p. 120.)

4. In contrast to the results with yeast and brain enzymes, it was found that hexokinases from rabbit muscle, rat muscle, or rat liver are separable into entities acting on fructose or glucose but not on both. Furthermore, cross inhibition by the sugars was not observed with the muscle and liver enzymes. The investigation did not include studies of the hexokinases from muscle and brain of the same individual, where the genetic constitutions of the tissues would be expected to be the same. Still, it is quite clear that the hexokinases from different sources can be much the same in unrelated species and quite different in different tissues of related or the same species. It is of course possible that the animal tissues contain several enzymes with overlapping specificities, each of which is produced under the action of a different gene. If true, this would provide a basis for one chemical step being under independent control of two genes. In any case, such results as these are not in accord with the idea that there is a simple and direct relation between single genes and single reactions. In addition the experiments provide further evidence that enzyme molecules do not have to be identical in order to catalyze the same biochemical reaction.

It has been mentioned previously that over 50 enzymes have been isolated in a crystalline form. Although crystallinity is a poor criterion of purity it does provide some basis for comparison of enzymes. One important problem is to determine whether all the molecules of such a highly purified material are identical. That is, does each catalytically active molecule that will act on one substrate have the same chemical composition and spatial configuration? With respect to enzymes with origins in different organisms or even different tissues in the same organism it is quite clear that the molecules are not necessarily alike.

When the enzyme is obtained from a single tissue the problem is much more difficult. Some results of investigations on crystalline

chymotrypsin are, however, rather suggestive. Since the isolation of this enzyme from beef pancreas by Kunitz and Northrop (457) numerous investigators have produced evidence that the enzyme is separable into at least six components all of which have similar or at least greatly overlapping catalytic specificities. These usually involve the hydrolysis of various proteins or certain simple peptides that contain phenylalanine, tyrosine, tryptophan, or methionine. These six components (B, α, β, γ, δ, and π-chymotrypsins) are distinguishable by one or more criteria which include solubility, mobility in an electrical field, relative rates of reactions with different substrates, and chemical compositions.

It is quite clear that all these components, and there are suggestions that more exist, are distinct but are closely related chemically. Although it cannot be stated that this evident multiplicity of molecular species is a general characteristic of enzymes or even that it is certain that the phenomenon is not an artifact of isolation, there seems little doubt that an enzyme need not have only one composition or total spatial configuration in order to maintain a given catalytic activity. (See p. 161 for further discussion.)

In addition to the above apparent natural variations in protein molecules, it should be recalled that it is possible to produce appreciable chemical alterations of enzymes without destroying the catalytic properties of the molecules. Herriott and Northrop (457) carried out experiments on the acetylation of pepsin by treating enzyme with ketene. The reagent reacts with $-NH_2$, $-OH$, and $-SH$ groups of the protein. Pepsin contains three or four free $-NH_2$ groups, and careful acetylation gives a crystalline triacetyl derivative that contains no detectable uncombined $-NH_2$ groups. This product is just as effective a catalyst as the original pepsin. Introduction of about 10 acetyl groups reduces the activity to 60%, and addition of 20 or more reduces the activity to less than 10%. It is probable that the reaction in the 60% active material is due to acetylation of the phenol group of tyrosine. These experiments and similar ones by other investigators provide further evidence that enzyme molecules contain relatively specific and non-specific portions, and that appreciable variations in composition and perhaps structure are permissible without appreciable alterations of catalytic activities.

2. Biological Synthesis of Macromolecules

It is presumed that the genes are macromolecules or conglomerants thereof, and it is presumed that the immediate products that are formed under the influence of genes are also macromolecules. Whether any small molecules take a direct part in the production of genes or their products is not known. In any event, until direct evidence to the contrary is obtained, it seems most reasonable to take the view that the same general principles apply in the biological synthesis and degradation of both small and large molecules.

It has already been pointed out that the presence of a simple molecule in a given concentration in a cell is dependent on a balance between the rate of formation and the rate of degradation of the substance. With a simple molecule there may be only one reaction that gives rise to the substance and only one reaction that degrades it. On the other hand complex molecules probably require many reactions in series or in parallel for synthesis or degradation. Still, it is reasonable to retain the concept that the nature of the product is a function of the concert of processes by which it is made and broken down. With macromolecules, where there exists the possibility of several functionally useful spatial configurations, it would appear that a given configuration can result entirely from the mode and order of synthesis and degradation, from the patterning influence of molecules in the environment as in antibody formation, or by a combination of both influences. All the aspects of the dynamic state must be included in an ultimate description of the biosynthesis of macromolecules.

This general viewpoint which emphasizes the interaction and interdependence of molecules in biological systems has been particularly well expressed by Claude (109):

> In the light of biochemical processes already known, it is conceivable that the duplication of essential and characteristic cell substances is the end result of a series of rigidly ordered chains of reactions, the final product in turn taking part at some point, and thereby directing the specificity of the same or other, interlocked, biochemical cycles. The specificity of a gene is not more striking and probably not more difficult to achieve, than the structural and functional specificity of a proteolytic enzyme, or that of a polysaccharide. Thus the term "self-duplication" appears meaningless when applied to those complex but highly organized cyclic biochemical processes leading to the production of new cell substances, and to the reciprocal action that these may exert on the system that produced them.

It follows from the view expressed above that the remarkable stability of chromosomal inheritance need be the result only of a very

severe restriction on the balance and complexity of interrelated reaction rates. Cellular geography may well be a very important factor both in synthesis and degradations, aiding in the persistence of substances that would be broken down faster than they would be formed if the cell were a sack containing a disorganized mixture of enzymes.

It should be emphasized that the preceding discussion is based on inference and not on experimental fact. Nothing is known of the mechanism of gene reproduction or of the mechanisms by which genes function directly. There is, however, a limited amount of information pertaining to the biological synthesis of enzymes.

It is possible that some enzymes are the immediate products of gene action, but it is quite certain that some are not. Largely as a result of the work of Northrop and his associates (457) it has been shown that a number of the enzymes that catalyze the hydrolysis of proteins exist themselves, in tissues, in the form of catalytically inactive proteins. These substances (zymogens) can be converted to enzymes by appropriate treatment with enzymes. Thus it appears that these substances are enzyme precursors, and they may represent intermediates in the biological synthesis of the enzymes to which they are converted. The best known of the zymogens are trypsinogen, chymotrypsinogen, and pepsinogen. All are proteins with quite similar elementary compositions. Pepsinogen has an appreciably higher molecular weight than pepsin, i.e., pepsinogen = 42,000 ± 3,000; pepsin = 38,000 ± 3,000.

Trypsinogen as isolated contains an inhibitor impurity that prevents the conversion of the zymogen to trypsin when it is present in a high enough concentration. If trypsin is added to trypsinogen, it is reported that the following reactions take place:

$$\text{Trypsinogen} \xrightarrow[\text{(or enterokinase)}]{\text{Trypsin}} \text{Trypsin}$$

$$\text{Trypsinogen} \xrightarrow{\text{Trypsin}} \text{Inert protein}$$

The reaction therefore appears to be autocatalytic but complicated by the secondary reaction. The conversion also occurs under the influence of another pancreatic enzyme, enterokinase, or by an enzyme from the mold *Penicillium*. High concentrations of magnesium and ammonium sulfates also speed the reaction. The nature of the change in converting the zymogen to the enzyme is not known, but it is thought to be due to hydrolysis of a peptide bond.

Chymotrypsinogen is converted to chymotrypsin by the action of trypsin, and this reaction is not autocatalytic. Jacobsen (313) has

provided evidence that at low temperatures one peptide bond is split to give π-chymotrypsin, which is 2 to 2.15 times as active as α-chymotrypsin. Further reaction splits successively one and three more peptide bonds to give δ- and α-chymotrypsins, respectively. The two apparent intermediates, π and δ, were not isolated, whereas the final product, α, was crystallized.

Pepsinogen can be converted to pepsin by acid, but it has been demonstrated that the catalysis by H^+ is not very effective. Experiments have been presented to show that the reaction is catalyzed much more effectively by the product, pepsin itself. During the course of the reaction about 15% of the protein nitrogen is split off from the pepsinogen, giving rise to pepsin and a basic polypeptide with a molecular weight of about 1,000. Possibly some individual amino acids are also removed. The polypeptide that is formed is an inhibitor for the action of pepsin, and it remains associated with the enzyme at *p*H values above 5.4. It is destroyed on long standing with pepsin, but it can be separated from the enzyme on the basis of solubility.

Particular note should be taken of several points of interest concerning the zymogens. First, it is unlikely that both the zymogen and the enzyme are immediate products of gene action. It is more likely that at least these enzymes are produced by a series of reactions perhaps starting with a direct product of gene action. Such a mechanism of course allows for a variety of indirect effects resulting from gene mutations. Next it should be noted that pepsinogen can give rise to two substances which can be of biological importance, pepsin and pepsin-inhibitor. It is not known whether the inhibitor has any other action than that of inhibition of pepsin, but any mutation leading to an increase or decrease in its rate of destruction could easily be interpreted as a direct effect on pepsin production. As a third point, the work with the zymogens further emphasizes that a small change in a macromolecule can make a very large change in its specificity.

Unfortunately there is little information on the biosynthesis of enzymes other than the kind discussed above. One of the reasons why progress in the field has been so slow is that methods for isolation and identification of medium-sized intermediates have not been available. With the widespread introduction of chromatography and counter current distribution methods this very important field should be expected to develop rapidly. A number of experiments that have been done are highly suggestive as to the specific nature of the problem. For example, Borsook and associates (62) have shown that radioactive amino acids are very rapidly incorporated by liver slices and homogenates into one

or more fairly large polypeptides. The amino acids are then transferred more slowly into the tissue proteins. These and related studies suggest that protein synthesis and the exchange of amino acids in proteins can take place by exchange of single amino acids or even fairly large peptides without loss of continuity of the protein molecules. There is no evidence as to what happens to the catalytic functions of enzymes during this dynamic interchange of amino acid components that certainly occurs *in vivo*. Presumably an enzyme as isolated in a pure form is made up of only those molecules that exist, at the time of extraction, in a sufficiently intact structure to show catalytic activity. For these reasons it is evident that an *in vivo* enzyme-catalyzed reaction must be more complex and must be influenced by more environmental factors than is a reaction in an isolated system where the catalyst itself is relatively stable.

A most elegant investigation of a change in the biosynthesis of a macromolecule that occurs as the result of a genetic change is that demonstrated in sickle-cell anemia by Pauling et al. (481). In the American Negro, red blood cells from about 8% of the individuals will assume odd sickle and other shapes if blood samples are placed in a sealed chamber for a period of 72 hours. A small percentage of the individuals (probably about 2%) have a severe and ultimately fatal anemia. The milder form of the disease does not appear to incapacitate the individuals that carry it. It is referred to as sickle-cell trait (or sicklemia); the more severe form is called sickle-cell anemia. According to Neel's (453) evidence both diseases are due to the action of a single mutant gene for which individuals with sickle-cell anemia are homozygous and those with sickle-cell trait heterozygous.

Investigations by Pauling, Itano, and associates (481) showed that sickling can be prevented by oxygen or carbon monoxide. Carbonmonoxyhemoglobins were prepared from blood of normal, sicklemia, and sickle-cell anemia individuals, and the electrophoretic mobilities of the proteins were compared. It was found that at *p*H 6.9 in phosphate buffer of 0.1 ionic strength, normal carbonmonoxyhemoglobin moves as a negative ion, whereas the corresponding substance obtained from sickle-cell anemia blood moves as a positive ion. Sicklemia blood contains both components (see Fig. 40) in ratios varying somewhat around 60% in the normal component. The difference that was found in isoelectric points (0.22) between the normal and the sickle-cell anemia carbonmonoxyhemoglobins corresponds to the existence of 2 to 4 more net positive charges per molecule in the abnormal protein than in the normal one. This difference is not due to the porphyrin

component of the hemoglobins since these substances, when isolated as the esters from the two kinds of hemoglobins, were shown to be identical. Electrophoretic studies show that the native globins from normal and sickle-cell sources are definitely different (265). This change in the globin portion, plus or minus oxygen, then evidently affects the structure of erythrocytes *in vivo,* a point that again em-

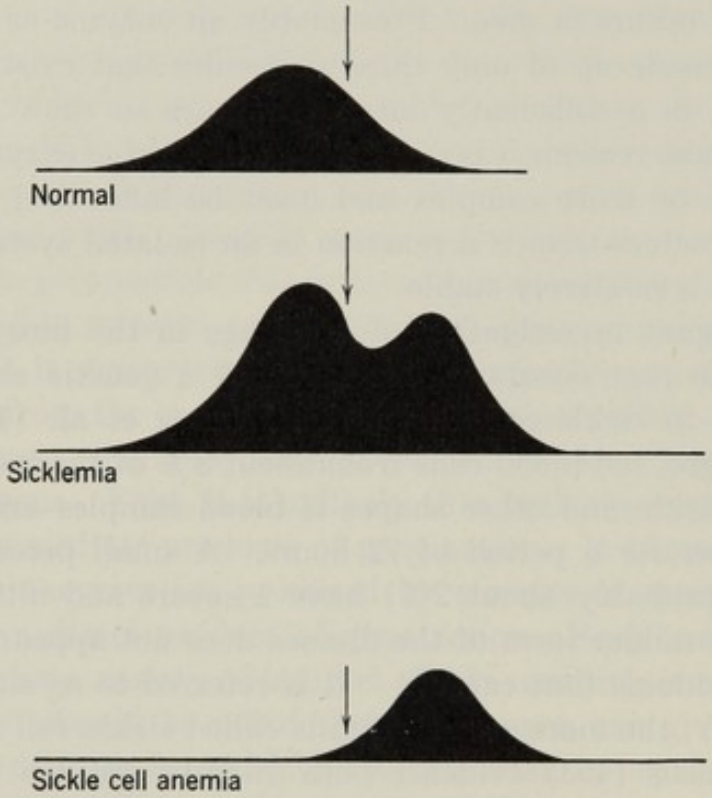


Fig. 40. Characteristics of carbon monoxyhemoglobins from normal, sicklemia, and sickle-cell anemia red blood cells. Scanning patterns from electrophoresis. After Pauling et al. (481).

phasizes the importance of the orientation of macromolecules with respect to each other.

In addition to the sickle-cell anemias discussed above, there are other known related inherited diseases which affect the composition of hemoglobin (311). Table 23 lists several of these and the type of hemoglobins found in persons afflicted with these diseases. The separation of the abnormal hemoglobins was accomplished by physical and chemical methods similar to those described for sickle-cell hemoglobin. It will be noted from the table that those individuals with sickle-cell anemia have fetal type (*f*) hemoglobin present as well as the *b* type characteristic of both sickle-cell trait and anemia.

In these examples and perhaps in the others that have been consid-

Table 23.* Types of Hemoglobins Characteristic of Several Human Diseases That Are Probably Inherited

Condition	Type of Hemoglobin Present				
	a (Normal Adult)	*b* (Sickle Cell)	*c*	*d*	*f* (Normal Fetal)
Normal adult	+	−	−	−	−
Normal newborn	+	−	−	−	+
Sickle-cell trait	+	+	−	−	−
Sickle-cell anemia	−	+	−	−	+
Hemoglobin-*c*-trait	+	−	+	−	−
Sickle-cell-hemoglobin-*c* disease	−	+	+	−	±
Hemoglobin-*d*-trait	+	−	−	+	−
Sickle-cell-hemoglobin-*d* disease	−	+	−	+	+
Thalassemia minor	+	−	−	−	±
Thalassemia major	+	−	−	−	+

* From Itano (311).

ered the potentiality for orientation must be an inherent property of the molecules themselves, but it is difficult to say whether the effect is a direct one or whether other kinds of molecules intervene in producing organized structures. These investigations on hemoglobin do demonstrate the kinds of changes in a macromolecule that can result from what are apparently single gene mutations (in sickle-cell anemia at least). The effect of such change does have deleterious effects on the organism, but it is not immediately fatal. The existence of an organism is not dependent, therefore, on one and only one chemical configuration for each macromolecule that is necessary for carrying on its metabolic processes. An appreciable amount of variation is permissible. In this example as in others it cannot be stated whether the variations arise directly as the result of gene action or indirectly by metabolic changes of gene products. That is, it seems possible that both the normal and the abnormal hemoglobins are intermediates in a series of reactions that occurs in all red cell primordia and that the effect of the mutation is to change the intermediate that is accumulated for erythrocyte formation.

3. Mutations and Single Enzymes

It is appropriate to state at the very beginning of this discussion of the experimental findings which relate mutational changes to changes

in enzymes that there is no experimental evidence which establishes that any one enzyme is a direct product of the action of any one gene. Some of the experimental work that is considered here can be interpreted to support the contention that such a direct relation does exist, but in each example alternative and equally plausible interpretations are possible. As stated previously, a major obstacle to reaching a decision on this point is that the genes and the enzymes themselves are not well enough understood on a biochemical basis, and, if it is to be considered that a single gene controls the formation of a single enzyme, it is necessary to have a complete understanding of the nature of single genes and single enzymes. Genes are not understood at all as biochemical units at the present time, and their definition as genetic units is still far from complete (see p. 241 on pseudoalleles). With regard to an adequate definition of a single enzyme, the discussions in Chapter 6 and the early part of this chapter have shown that this too is extremely difficult. Some of the reasons for this difficulty can be summarized as follows:

1. An enzyme which can be isolated and characterized by physical and chemical criteria *in vitro* is not necessarily identical with the same enzyme *in vivo*. In a cell it may exist in combinations with other enzymes in a fashion that is essential to its functions. In addition, in a cell, it undergoes a dynamic interchange of its component parts with similar parts in the biochemical environment.

2. Macromolecules with the same or similar catalytic functions are sometimes different even though they may be obtained from the same tissue. An enzyme from different tissues with the same genetic constitution can have different catalytic specificities and physical and chemical properties as discussed above.

In addition to these problems that have to do with the nature of genes and enzymes, interpretations of experimental findings that relate these two kinds of physiological units are also dependent on an understanding of the interdependence and balance of biochemical reactions that occur in the cells under consideration. It is not an acceptable simplification to focus attention entirely on a single enzyme and a part of a reaction that it may catalyze without giving attention to all the components of the reaction. That is, all the reacting substances, including the catalyst, and all the products must be known before the physiological results of a mutation can be understood and before the relations of genes to enzymes can be defined.

As a basis for a practical evaluation of the experimental work that has been done on gene enzyme relations an attempt has been made to summarize the more significant factors that can influence the course of

a reaction. This summary is given in Fig. 41. It is obvious that if mutation destroys a primary gene having to do with production of *E* (Fig. 41) the enzyme will not be produced in the organism, and the reaction will not take place unless catalysts of a different origin are available. This represents the most extreme single result of a mutation, and it may occur when a gene is lost through a chromosomal deficiency. On the other hand, other factors besides enzyme loss can influence a reaction rate to any degree from reduction of the rate to zero to increasing the rate to some maximum allowable by the metabolic environment of the reaction. It follows that an analysis of the effect of a mutation on an enzyme can be extremely complex when it is the catalytic property of the enzyme that is utilized as a basis for comparisons.

For example, consider the effect of a mutation that results in the failure of growth of an organism because a reaction such as that illustrated in Fig. 41 does not take place. In such an instance it might be that the enzyme is not produced because a primary gene product is not produced, but it could also be that the enzyme is altered by action of the primary or the secondary genes, so that (1) substances or forces controlling the orientation of the enzyme in the cell are not produced, or (2) the substrate is not formed rapidly enough, or (3) an inhibitor for the reaction is produced. In addition to these possible changes in reactants, the change in reaction rate could be due to an alteration in a coupled reaction or in the alternate reactions that affect the enzyme, substrate, or inhibitor as shown in Fig. 41. Finally, the reaction may not take place significantly because of some combination of these influences. A limited or otherwise altered growth rate can be considered in the same light except in the case of loss of the primary gene. It should be noted at once that, in the description of experimental findings that follows, no examples will be found that can be placed with assurance in any one of the categories suggested above (or in Fig. 41). It is quite clear, however, that a good many of the examples cannot be placed in the category represented by loss of an enzyme as a direct result of the loss of a primary gene since it is shown that several of the catalysts investigated are not missing from the tissue.

In considering these experimental examples it is somewhat tempting to class them on an arbitrary basis of apparent directness or indirectness of the genetic effect on the enzyme activity, that is, to call the example a direct effect when a mutation causes reduction of an enzyme activity below that detectable by available analytical methods. Such a classification is not justifiable for at least one important reason.

Even when dealing with an enzyme with a very high turnover number most analytical methods will not detect less than 10^{10} molecules of enzyme per milliliter. Thus it is not possible to state that an enzyme is missing, only that it must be present in a concentration less than that which can be determined by the method of analysis. A classification like that suggested above is even less justifiable when it is

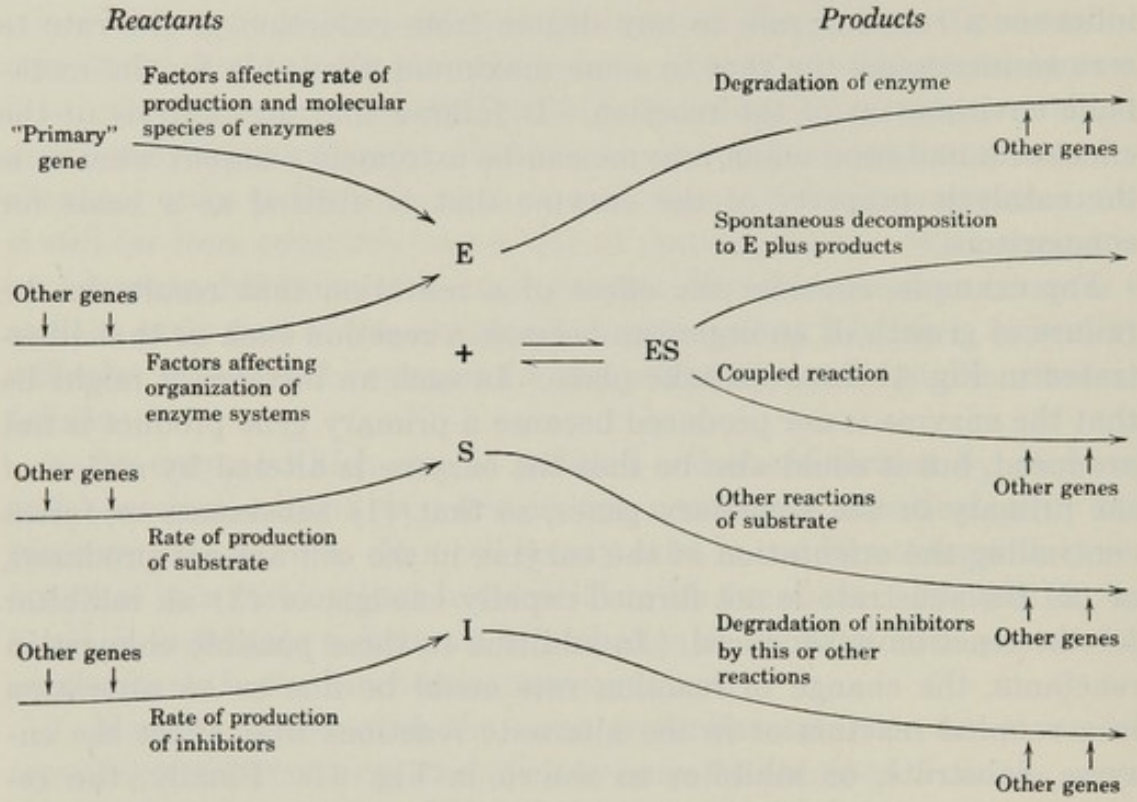
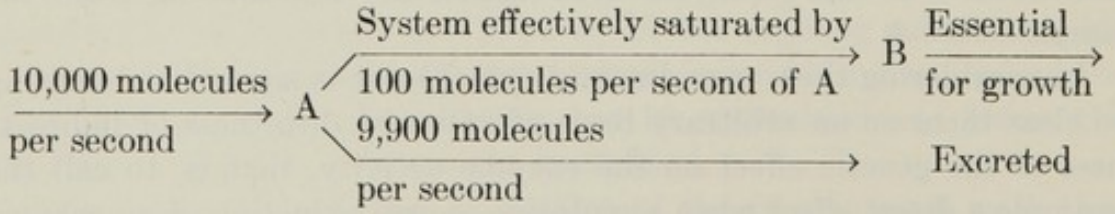


Fig. 41. A summary illustrating some factors of genetic origin that influence reaction rates.

applied to phenotypic effects of mutations other than those based on enzyme activities. To be specific, in a hypothetical example where growth rate is used as a criterion for the effect of a mutation, assume that an organism normally produces a substance A at the rate of 10,000 molecules per second. A is converted to a substance B that is required for growth and it is also excreted as indicated in the diagram. If the

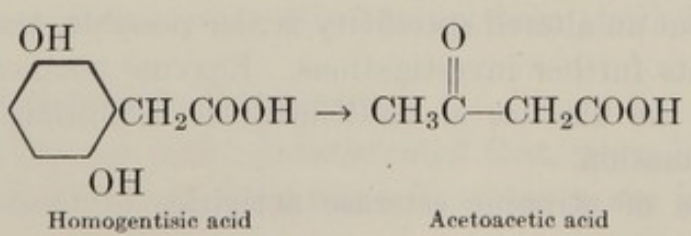


enzyme system producing B is effectively saturated by 100 molecules

per second of A then the production of A can be reduced 100-fold by genetic changes without affecting the growth rate of the organism significantly. On the other hand a further 10-fold reduction in the rate of formation of A, a change of smaller magnitude than the first, may well reduce the growth rate of the organism to a negligible value. Few if any of the experiments that have been carried out have been directed at the analysis of such phenomena as this. Still, examples that appear to be similar in principle have been described. In Chapter 8 note the results of introducing certain mutant genes on the accumulations of compounds by the adenine (p. 207), the uridine (p. 210), and the histidine (p. 200) mutants of *Neurospora*.

The Enzymatic Degradation of Homogentisic Acid in Man

Garrod (192) described a human metabolic disorder known as alcaptonuria which does not handicap the individuals affected but causes their urine to turn black on exposure to air. Evidence was provided that this characteristic is inherited as a single recessive gene. The substance in the urine of alcaptonurics that gives rise to the black pigment is 2,5-dihydroxyphenylacetic acid, or homogentisic acid, and it was found that if this substance is fed to normal individuals it does not appear in the urine. Gross (233) investigated this phenomenon on an enzymatic basis using blood sera from a number of normal and alcaptonuric individuals. His experiments in 1914 probably represent the first carried out in attempts to relate enzyme constitution to inheritance. It was found that homogentisic acid disappeared when it was added to serum from a normal individual but not when it was added to serum from an alcaptonuric individual. It can be estimated from the data given that a reaction rate equivalent to about 2% of the normal concentration would have been detected by the analytical method utilized in the experiments, and so it is a justifiable conclusion that the enzyme activity of an alcaptonuric serum is less than 2% of the activity of the enzyme in a normal serum. Other tissues than blood were not examined for enzyme activity, nor has evidence been provided that the difference is not due to the presence of inhibitors. The reaction itself is not well understood, but it is presumed to be as follows:

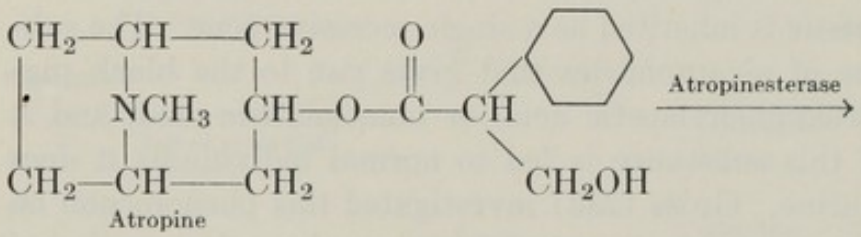


Homogentisic acid Acetoacetic acid

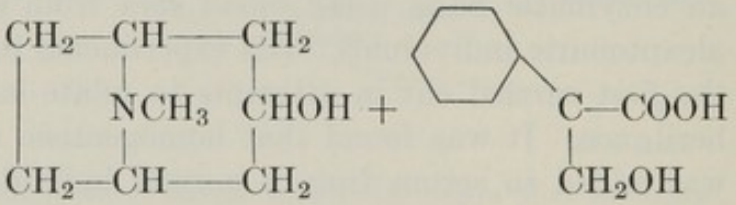
This is equivalent to a hydrolysis giving 2 molecules of acetoacetic acid, but the stoichiometry of the reaction is not known and it is most likely that more than one step and more than one enzyme are involved. Thus the exact nature of this metabolic disorder is not known, and a reinvestigation of the problem would be of interest.

Some Alkaloid Esterases in Animal Tissues

It has been observed by a number of investigators that the blood from some rabbits can hydrolyze the alkaloid atropine, and genetic experiments gave evidence that this property is inherited as a partially dominant character (44). Glick and associates (200, 538) carried out extensive experiments on the occurrence and the specificity of the enzyme involved in this hydrolysis. They also investigated the kinetics of the reaction and determined an enzymes-substrate dissociation constant for the system. The reaction involved is shown herewith. The



Atropine



enzymes from the blood of animals which give the hydrolytic action act upon various salts of atropine, or homoatropine, novatropine, cocaine, tropacocaine, scopolamine, and acetylcholine as shown in Table 24. The results suggest that more than one enzyme is involved in these hydrolytic reactions, and it has indeed been established that atropine esterase and acetylcholine esterase are different, as might be expected from the great difference in the chemical structures of the substrates. Since horse serum and the serum from the atropine esterase negative rabbit II show hydrolysis of cocaine and tropacocaine a third enzyme is suggested, but an altered specificity is also possible, and the question certainly merits further investigations. Enzyme purifications as well as studies on the kinetics of multisubstrate inhibitions should yield valuable information.

Comparisons of atropine esterase activities of tissues other than

Table 24. Hydrolysis of Tropine Esters *

Compound	Enzymatic Hydrolysis (Tropine Esterase Units per 0.1 Ml of Serum) 1% Substrate			Corresponding Non-Enzymatic Hydrolysis
	Horse	Rabbit I †	Rabbit II †	
Atropine sulfate (L-hyoscyamine)	0	96	0	10
L-Hyoscyamine hydrobromide	0	115	0	10
Homoatropine hydrobromide	0	64	0	33
Novatropine	0	14	0	40
Cocaine hydrochloride	0	50	34	148
Tropacocaine hydrochloride	146	11	15	12
Scopolamine hydrobromide	0	72	0	9
Acetylcholine chloride	1,510	150	170	130

* From Glick and Glaubach (200).
† Corrected values.

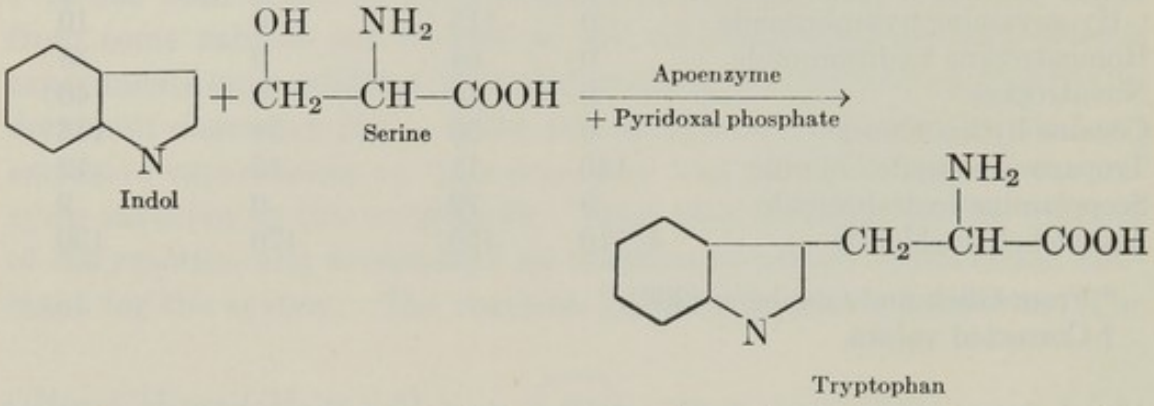
blood were made from both positive and negative animals. Some activity was found in all preparations from the positive rabbits, the serum, liver, and intestinal mucosa activities being particularly high while brain and gastrocnemius muscle were low in activity. In contrast to these results the animals which showed atropine esterase negative blood also showed atropine esterase negative tissues. In addition mixing sera from positive and negative animals yielded enzyme preparation with an activity equivalent to that of the positive portion. Thus, if the lack of esterase activity in the negative animal is due to inhibitors, the inhibition is a fortuitously balanced one. It is difficult to estimate, from these experiments, the maximum relative amount of enzyme that could be present in the esterase negative animals since there is a slow spotaneous hydrolysis of the substrate. However, this value appears to be less than 1% of the normal figure.

Some other points of interest concerned with these investigations rest on the observations that atropine esterase activity is found in the livers of cats, rats, dogs, and frogs, but it is not found in sera from these animals. Guinea pig liver or serum preparations do not act on atropine, but the liver contains a potent homatropine esterase. The inheritance of these characteristics apparently has not been studied.

Tryptophan Synthesis in *Neurospora*

Tatum and Bonner (638) demonstrated that, when living but non-growing mycelial pads of the fungus *Neurospora* are incubated with

indol, tryptophan is formed. The yield of the amino acid is increased by addition of serine. Subsequently, Umbreit, Wood, and Gunsalus (660) prepared a cell-free enzyme preparation from *Neurospora* and showed that it contained an enzyme (now called tryptophan desmolase or tryptophan synthetase) that catalyzes tryptophan formation from indol and serine. Pyridoxal phosphate is required as a coenzyme for the reaction as shown.



Although it appears that this reaction is a simple one of removal of the elements of water to effect the coupling, it is more probable that more than one step and perhaps more than one enzyme is involved. Lein et al. (362) and Mitchell and Lein (423) isolated a mutant strain of *Neurospora* (C83) that will utilize tryptophan for growth, but it will not use indol as do nearly all the other tryptophan mutants of this fungus (see p. 194). Genetic tests provided evidence that strain C83 differs from the wild-type strain of the mold by a change at a single genetic locus, so far as a tryptophan requirement is concerned. Further tests showed that, while cell-free preparations containing soluble enzymes from wild and several *Neurospora* mutants contain a highly active system for tryptophan synthesis from indol plus serine, similar preparations from strain C83 gave no measurable activity. The methods for analysis allow for maximum activities of about 0.2 to 0.3% of those found in active strains. A mixture of normal and mutant enzyme preparations shows activity corresponding to the proportion of the normal system. Dialyses and purifications of normal and mutant preparations by ammonium sulfate fractionation and by absorption and elution from calcium phosphate yielded the same result found with crude extracts.

Yanofsky (715, 716) extended these investigations, using another mutant (Y1952) which was shown to be either closely linked to, or allelic with, strain C83. This strain is similar to C83 in its growth

requirement, and the enzyme activity was not found in the cell-free extracts. In addition it was observed that a gene, the suppressor of Y1952, *su*-Y1952, at another locus acts as a partial suppressor (see Chapter 10) of the mutation in Y1952, whereas it has no such action on C83. That is, the double mutant *su*-Y1952 will grow to a considerable extent in the absence of tryptophan, and produces the tryptophan-synthesizing enzyme system which can be found in the cell-free preparation from the strain. These experiments with *Neurospora* strains C83, Y1952, and the suppressor of Y1952 show an apparently perfect correlation between a growth requirement and a deficiency in an enzyme activity as the result of gene mutations. However, as already pointed out, such a seemingly simple direct relation between a gene, an enzyme, and a reaction may be superficial, and each component of the system must be examined rigorously.

This important point is emphasized by the results of investigations of Hogness and Mitchell (285) on the inheritance of the tryptophan desmolase system in *Neurospora*. It has been demonstrated that certain strains which do not require tryptophan for growth can be quite as deficient in the enzyme activity as mutants C83 and Y1952, when they are grown under appropriate environmental conditions. One of these strains was derived from a cross of C83 to wild type. An analysis of 18 asci showed a 4 to 4 segregation of the parental types with respect to tryptophan requirement, as expected. But one ascus yielded six cultures instead of four deficient in the enzyme activity. A further examination showed that activity could be obtained in preparations from the aberrant wild-type pair of cultures by using young mold grown in the presence of tryptophan or mold grown in the absence of tryptophan (Fig. 42). Strain C83 cannot be grown in the absence of tryptophan and it does not yield tryptophan desmolase activity even in very young cultures or at low levels of tryptophan, but it is possible that the presence of tryptophan has as direct an influence on the enzyme deficiency in this strain as does the particular gene mutation it carries.

Another type of tryptophan desmolase deficiency was encountered (285) in the analysis of progeny from a cross of a histidine-requiring strain of *Neurospora* (C84) to wild type. The original C84 culture was observed to have 2 to 3 times as much tryptophan desmolase activity as wild type, but as shown in Fig. 43 some of the histidine-requiring progeny were found to have a high enzyme content whereas others were deficient in this activity at intermediate stages of growth. The interaction of the gene which results in the histidine requirement with at

least one other appears to control the high and low levels of tryptophan desmolase activity.

In further experiments with the tryptophan desmolase system it was demonstrated (285) that strain C83 could be induced to back-mutate

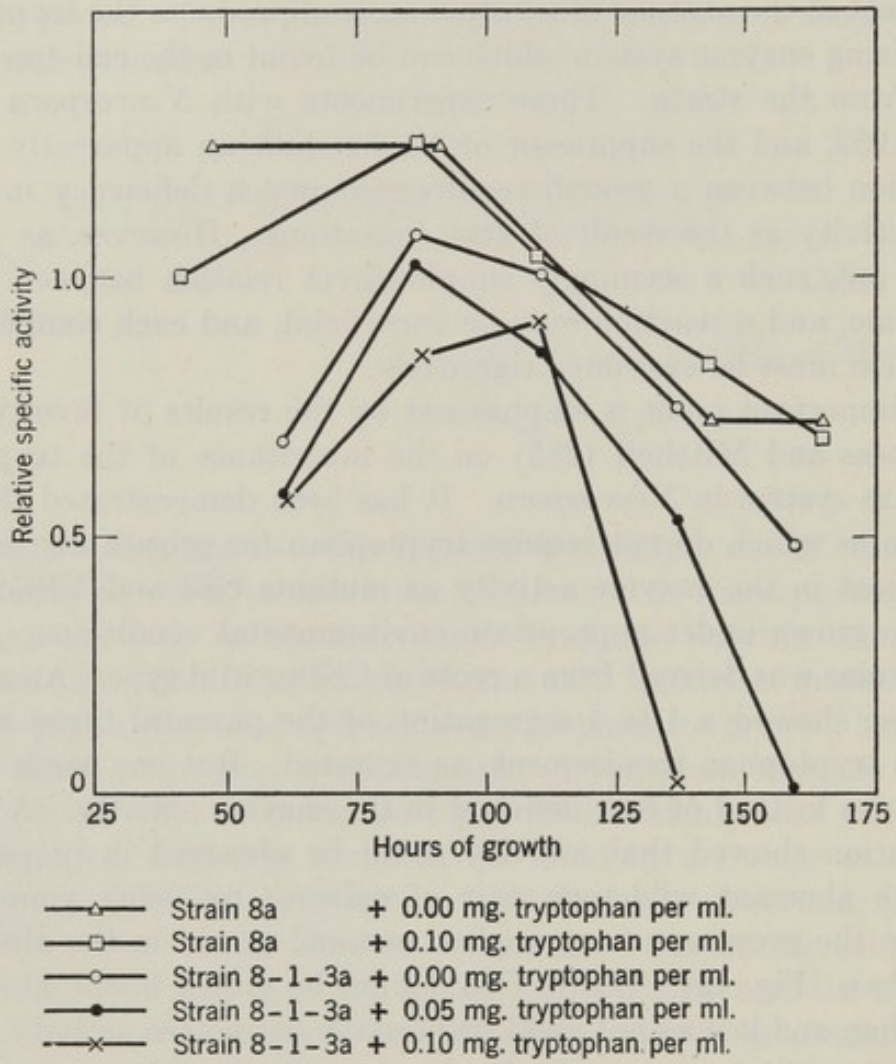


Fig. 42. The relative specific activity of tryptophan desmolase in two phenotypically wild strains of *Neurospora*. The time of loss of the enzyme activity by strain 8–1–3a in the presence of tryptophan is variable, occurring sometimes in less than 96 hours.

to tryptophan independence by ultraviolet radiation. Colonial and albino markers were used to insure detection of contamination. After a 20-fold purification of tryptophan desmolase from wild type and from the back-mutated strain the preparations were compared by determining apparent affinity constants of each for indol, serine, and pyridoxal phosphate. These results and those of Yanofsky on the suppressed mutant *su*-Y1952 (p. 260) are summarized in Table 25. Considering

Table 25. Apparent K_s Values for Tryptophan Desmolase. Measured at 35 or 37° in Phosphate Buffer *p*H 7.6–7.8 *

Substrate or Coenzyme	K_s	K_s
Indol	—	2.3×10^{-5}
L-Serine	3.4×10^{-3}	6.3×10^{-3}
	6.0×10^{-3}	
Pyridoxalphosphate	3.0×10^{-6}	3.4×10^{-6}

* Data from Hogness and Mitchell (285).

experimental errors and slightly different conditions it seems unlikely that there is a significant difference in the values obtained for these semi-empirical constants whether the enzyme was obtained from wild type, the suppressed Y1952, or the back-mutated C83. Significant differences may exist, however, within the limits of experimental errors.

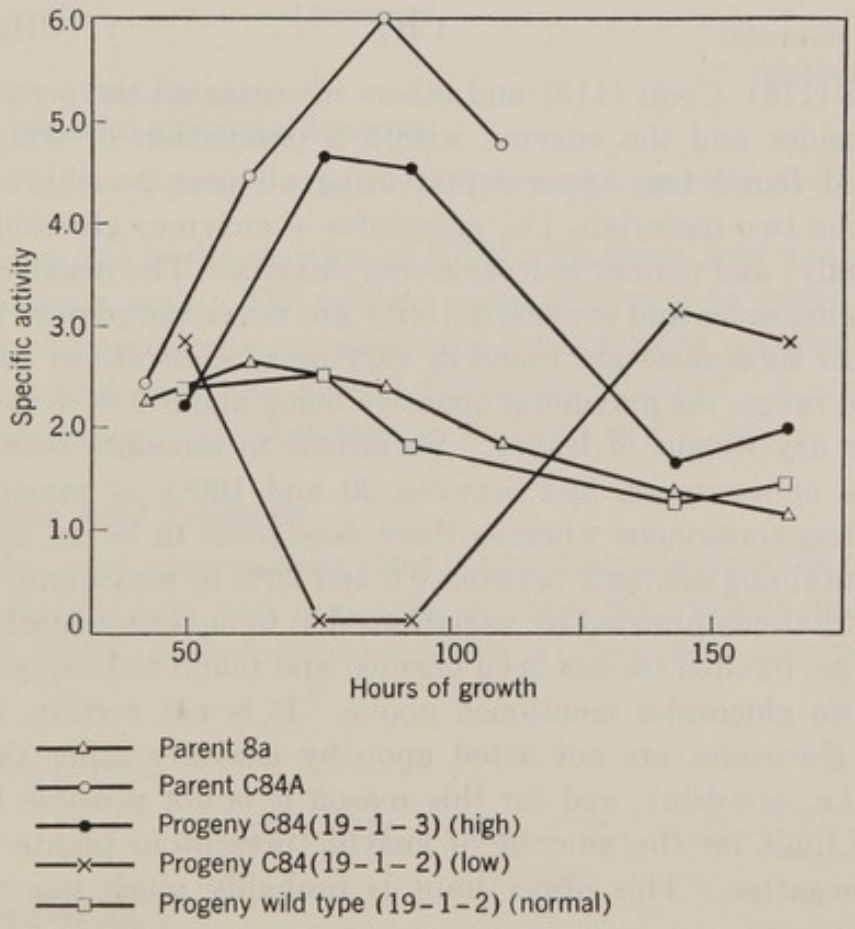
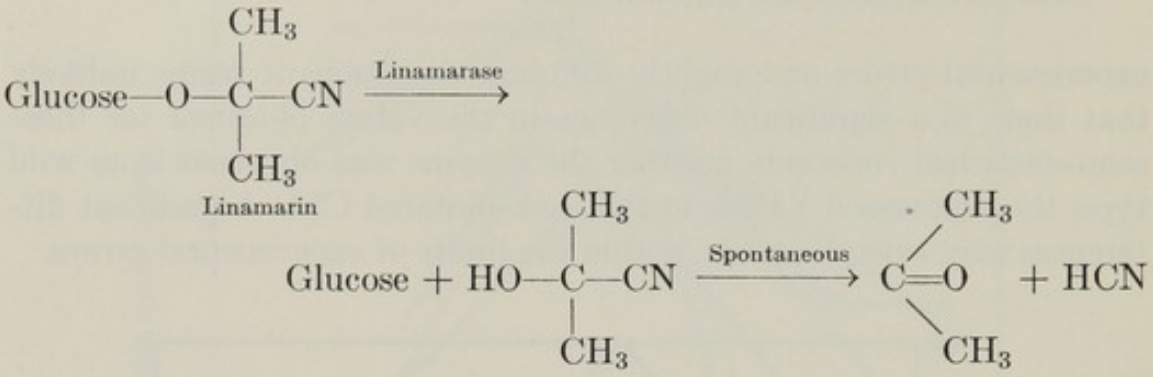


Fig. 43. Segregations of tryptophan demolase activity in a cross of wild-type 8a × C84A (histidine-requiring) strains of *Neurospora*. Many of the strains showing the low type of activity (— × —) either did not regain activity at all after it was lost, or they regained it in a longer time than in the example shown.

Linamarases in White Clover

It has been recognized for many years that white clover (*Trifolium repens*) contains substances that yield hydrocyanic acid when they are acted upon by an enzyme that is sometimes present in the same plant. The two substances that have been isolated, linamarin and lotaustralin, are glucosides of the cyanohydrins of acetone and methylethylketone respectively. The cyanide producing reaction with linamarin is shown.



Corkill (116), Coop (115) and others investigated the occurrence of the glucosides and the enzyme within a population of white clover plants and found four types representing all four possible combinations of the two materials, i.e., glucosides + enzyme; glucosides only; enzyme only; and neither glucosides nor enzyme. The negative classes for both glucosides and enzyme activity are never completely negative. The former substances are found in varying concentrations over about a 150-fold range, the maximum quantity being about 0.04% in terms of HCN per dry weight of leaves. Variations in amounts between 0.66 and 6.6% of maximum and between 20 and 100% of maximum are more or less continuous whereas there does seem to be an absence of plants containing amounts between 6.6 and 20% of maximum. Results are somewhat complicated by variations due to environmental changes. The enzyme linamarase has been purified and found to be quite specific for the two glucosides mentioned above. It is not certain, however, that the glucosides are not acted upon by enzymes other than linamarase (i.e., emulsin), and for this reason it is not possible to define an upper limit for the amount of enzyme present in plants listed as enzyme negative. This upper limit is probably much less than 1% of the maximum.

Atwood and Sullivan (9) and Corkill (116) obtained evidence that the presence of glucosides and of linamarase is each determined by non-allelic dominant genes (*Li* and *Ac*) carried in a clover population. Although it is possible to measure the action of linamarase in a quan-

titative manner by the rate of appearance of glucose, HCN, or ketone it was not practicable to do so in the large numbers required for the genetic tests. Consequently a crudely quantitative colorimetric method for HCN determination was applied. Clover leaves, leaves plus glucoside, or leaves plus enzyme were placed in test tubes containing picric acid paper suspended above the reaction mixture. The intensity of

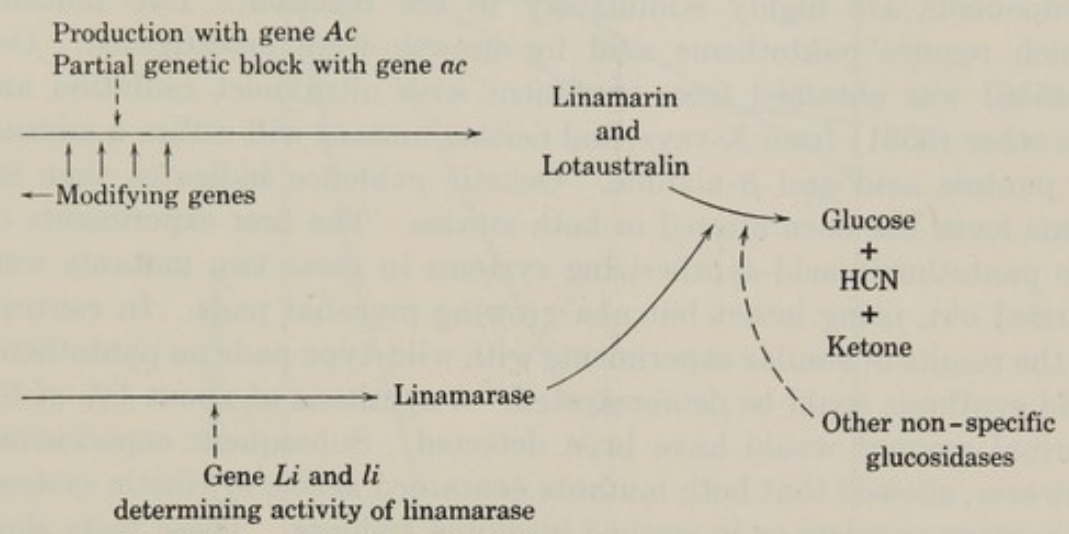
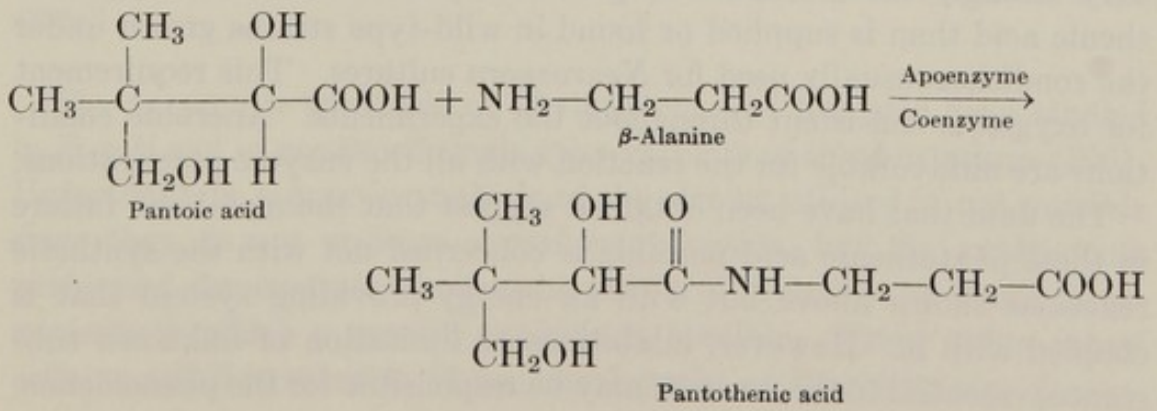


Fig. 44. Genetic control of HCN formation in white clover.

color change due to the reaction of HCN with picric acid was estimated on an arbitrary scale. The method seems adequate for rough analysis, but more elaborate ones are necessary for obtaining an adequate biochemical definition of the genetic differences involved. A summary of the clover cyanogenetic phenomenon is shown in Fig. 44.

Pantothenic Acid Synthesis

Neurospora produces an enzyme system that catalyzes the condensation of pantoic acid and β-alanine to form the vitamin pantothenic acid (673, 672). An unknown coenzyme is presumably required for the reaction.



Actually the mechanism of this reaction is not known, and it would be not unexpected if a coupled reaction is required to provide the energy for formation of the peptide bond. The enzyme system has been demonstrated in intact non-growing mycelium and in acetone dried powder from wild-type *Neurospora*. The system has not been prepared in a soluble form, but it has been demonstrated that soluble components are highly stimulatory to the reaction. Two mutants which require pantothenic acid for growth were investigated. One (34556) was obtained from treatment with ultraviolet radiation and the other (5531) from X-rays, and neither mutant will utilize a mixture of pantoic acid and β-alanine. Genetic evidence indicates that the same locus has been altered in both strains. The first experiments on the pantothenic acid-synthesizing systems in these two mutants were carried out, using intact but non-growing mycelial pads. In contrast to the results of similar experiments with wild-type pads no pantothenic acid synthesis could be demonstrated. A synthesis of about 1% of the normal amount would have been detected. Subsequent experiments, however, showed that both mutants contained active synthetic systems in acetone powders or in washed insoluble residues. These facts show that in the three stages of isolation of the enzyme system, in intact mycelium, or acetone powder, the capacity for synthesis progressively increases with the mutants but it is not particularly altered with the wild type. The initial difference is extreme, showing almost as complete a lack of enzyme activity by the mutant as has been demonstrated in any experimentally related example. On the other hand, at the final state of purification attained there is no significant difference in the capacity for pantothenic acid synthesis between the mutant and wild-type preparations. Some additional significant experiments (534, 674) have shown that it is possible to alter environmental conditions in such a way that the pantothenic acid mutants will synthesize pantothenic acid. If the vitamin is supplied and the cultures are aerated very strongly the mutants will grow and produce much more pantothenic acid than is supplied or found in wild-type strains grown under the conditions usually used for *Neurospora* cultures. This requirement for oxygen is consistent throughout the experiments. Anerobic conditions are unfavorable for the reaction with all the enzyme preparations.

The data that have been obtained suggest that the metabolic failure in these pantothenic acid mutants is concerned not with the synthetic reactions shown above but with an energy-providing system that is coupled with it. However, inhibitions or limitation of unknown substances essential to the reaction may be responsible for the phenomenon.

In any case it seems clear that it is not the presence of the catalyst most obviously concerned with the final step in synthesis of the vitamin that is determined directly by the mutant genes of these pantothenic-less strains.

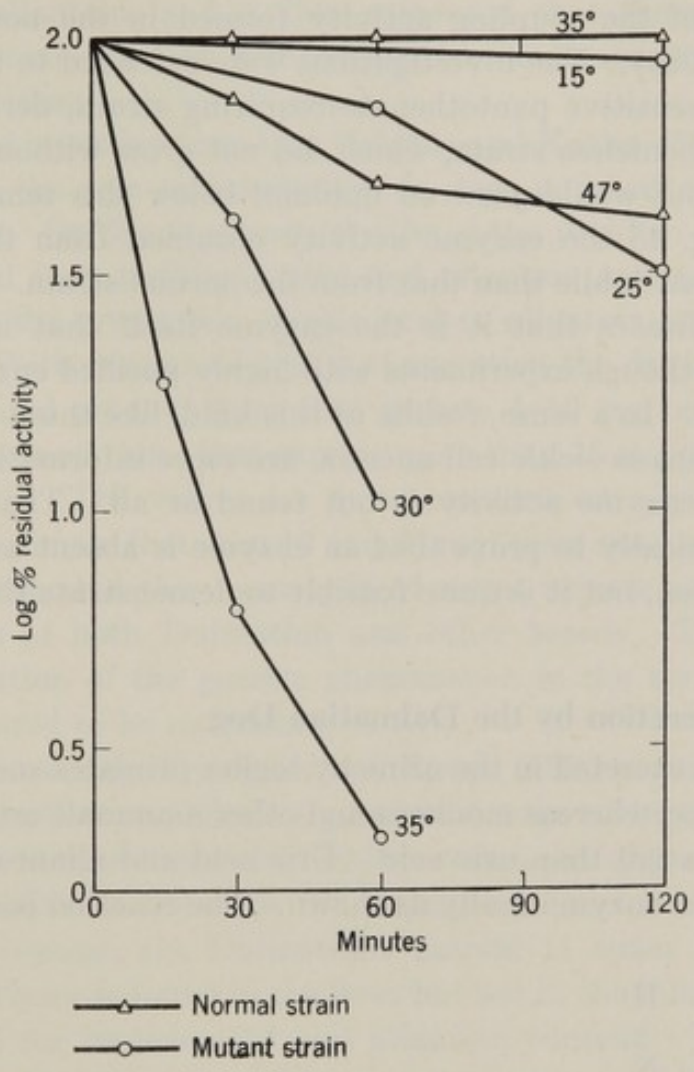


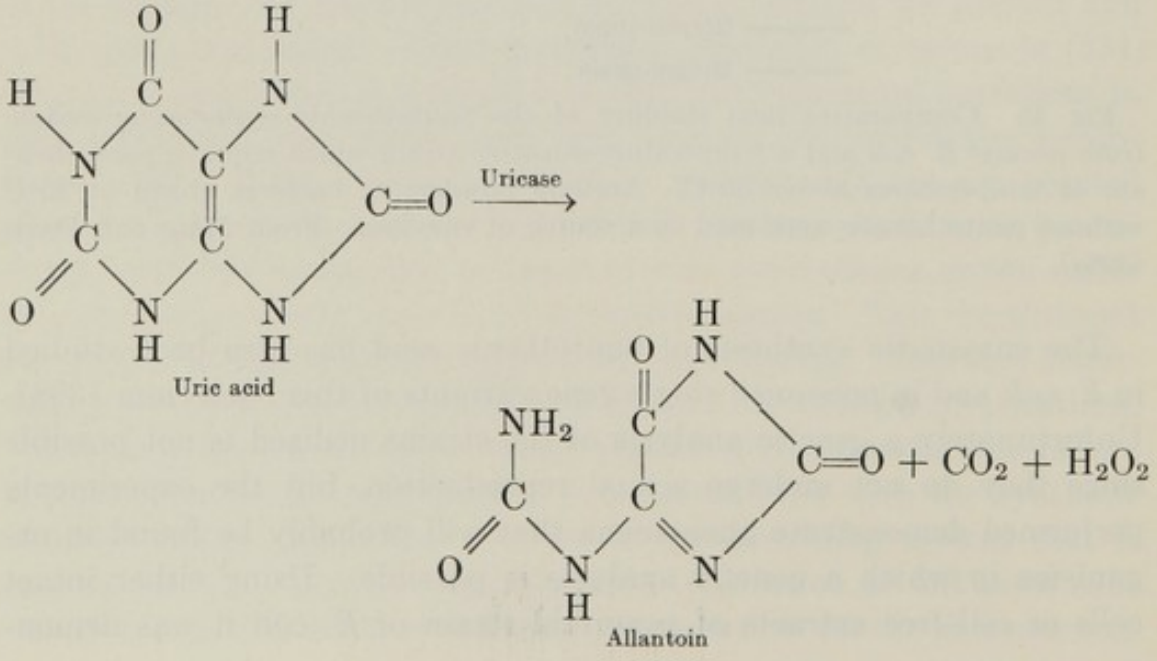
Fig. 45. Comparative heat stability of the pantothenate-synthesizing system from normal *E. coli* and a temperature-sensitive strain which requires pantothenate at temperatures above 30°C. Acetone powders of bacteria grown at 25°C without pantothenate were used as a source of enzymes. From Maas and Davis (398*a*).

The enzymatic synthesis of pantothenic acid has also been studied in *E. coli* and in presumed single gene variants of this bacterium (398). Unfortunately a genetic analysis of the strains utilized is not possible since they do not undergo sexual reproduction, but the experiments performed demonstrate phenomena that will probably be found in organisms in which a genetic analysis is possible. Using either intact cells or cell-free extracts of a normal strain of *E. coli* it was demon-

strated that an enzyme system was present which could effect the coupling of pantoic acid and β-alanine to produce pantothenic acid. ATP is required in the reaction. A variant strain of the organism which requires pantothenic acid for growth was found to have less than 0.05% of the coupling activity formed in the normal parental bacterium (398*a*). The investigations were extended to the study of a temperature-sensitive pantothenate-requiring strain, derived from the above pantothenicless strain, which did not grow without the vitamin above 30°C but would grow on minimal below this temperature. As shown in Fig. 45 the enzyme activity obtained from this variant is much more heat-labile than that from the normal strain. The evidence presented indicates that it is the enzyme itself that is altered and more labile although experiments with highly purified enzyme have not been reported. In a sense, results of this kind, like those obtained with the hemoglobins in sickle cell anemia, are more informative than those in which an enzyme activity is not found at all. That is, it is not possible technically to prove that an enzyme is absent as the result of a gene mutation, but it is quite feasible to demonstrate that an enzyme is altered.

Uric Acid Excretion by the Dalmatian Dog

Uric acid is excreted in the urine by higher primates such as man and the chimpanzee, whereas monkeys and other mammals excrete allantoin to a greater extent than uric acid. Uric acid and allantoin are related chemically and enzymatically as shown. The reaction is a complex one



involving several chemical steps. The enzyme has been highly purified, and there is no evidence that more than one enzyme is required. In 1916 Benedict (42) made the observation that Dalmatian dogs, unlike other dogs and carnivores in general, but like man, excrete uric acid rather than allantoin, and it thus appeared that there might be a heritable difference in dogs with respect to the presence or absence of the enzyme uricase.

A number of investigations (see Trimble and Keeler (657)) provided evidence that the uric acid excretion in dogs is inherited as a single recessive gene. On the biochemical side of the problem it was shown that, in actual fact, both uric acid and allantoin are excreted by all dogs but that the proportion of uric acid to allantoin is higher in the Dalmatian. With respect to uric acid excretion the dogs fall into two clearly separated groups, those that excrete 4–10 mg per kg of body weight per day, and those that excrete more than 28 mg per kg of body weight per day. The values are quite constant on a given diet, and intermediate animals (between 10 and 28 mg) were not found. Investigations demonstrated the occurrence of equal uricase activity in the livers of dogs of both Dalmatian and other breeds. Thus a simple direct explanation of the genetic phenomenon in the terms of uricase activity appeared to be untenable.

Much more recently Friedman and Byers (185) provided some experimental data that contribute a great deal to an understanding of the problem. It was found that the total excretion per unit of time of uric acid plus allantoin is the same for Dalmatians as for other dogs but that, on the average, the Dalmatians excrete 11 times as much uric acid. Since uricase is found in the liver but not in the kidney, the blood was examined for its uric acid and allantoin content. The following average figures in terms of milligrams of nitrogen per 100 ml of blood plasma were obtained:

Dalmatians	Uric acid	0.20
	Allantoin	0.23
Other dogs	Uric acid	0.11
	Allantoin	0.33

Here as in the case of the products excreted the totals are the same but the differences in relative proportions of the two substances are much higher in the urine. This apparent anomaly was resolved by studies of renal clearances. It was found that uric acid is passed into the

glomerular filtrate of the kidneys, but in the Dalmatians it is not reabsorbed in the kidney tubules whereas in other dogs it is reabsorbed to a great extent. Allantoin is not reabsorbed in the kidney of either type of dog.

It seems apparent, therefore, that the phenomenon of uric acid excretion by Dalmatians is due primarily to a fault in the mechanism of reabsorption in the kidney and not to a fault in the production of uricase in the liver. In other dogs the reabsorbed uric acid is further

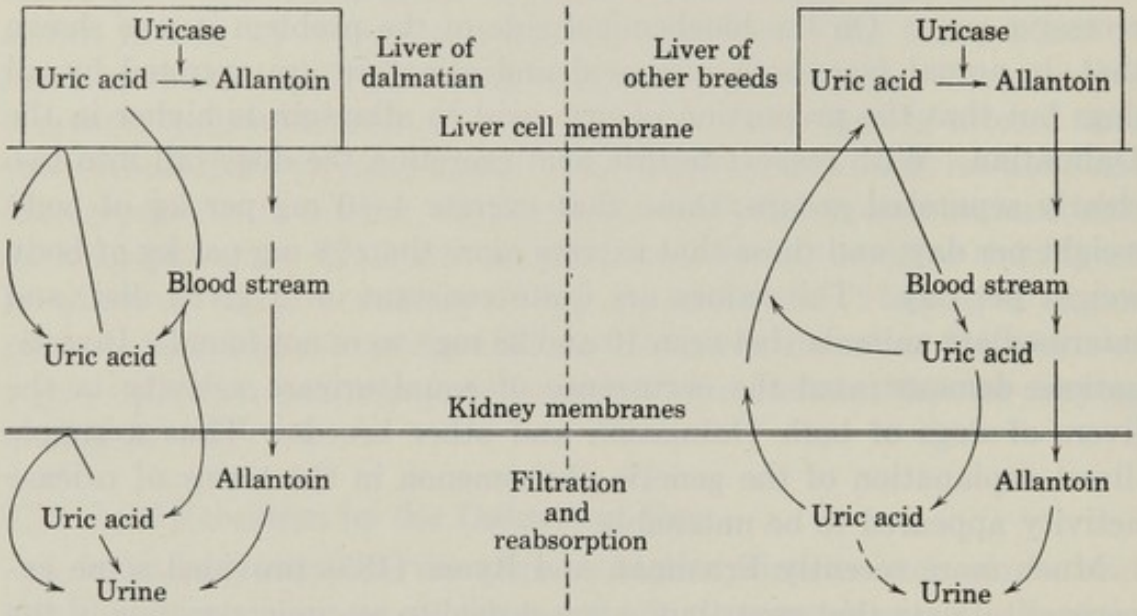


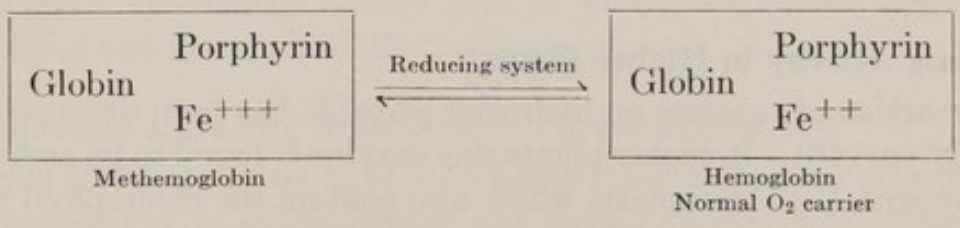
Fig. 46. Excretion of uric acid and allantoin by Dalmatians and other dog breeds. Uric acid is not reabsorbed through the kidney membrane and possibly not through the liver membrane in the Dalmatian.

converted to allantoin when carried back to the liver. There still remains one point that is not entirely clear. From the data on the blood composition with respect to uric acid and allantoin that are given above and from experiments by Friedman and Byers on the blood composition after tying off the ureters it appears, superficially, that Dalmatians have a less effective uricase system than other dogs. Rimington (515) has accounted for this apparent difference by assuming that reabsorption by liver cells is faulty in Dalmatians just as has been demonstrated for kidney tubule cells. The two systems discussed above are diagrammed in Fig. 46. It is now quite clear that this single gene difference in dogs does not necessarily have anything to do with the production of the enzyme uricase. It may have to do with the formation of structural materials directly or indirectly or with some enzymatic process concerned with the transport of uric acid through cell membranes.

These results provide a good illustration of the complexities encountered in the analysis of the results of a genetic change. Obviously the simplest interpretation of the original observations is inadequate, and indeed the simplest interpretation of results in general is acceptable only as a basis for further experimentation.

Idiopathic Methemoglobinemia in Man

Idiopathic methemoglobinemia is a comparatively rare disease of man, and it is considered by Gibson (194) to fall in the same category of heritable metabolic disorders as alcaptonuria and cystinuria. The genetic data in this case appear to be limited to observations that the disease occurs in families rather than at random in the population, but the biochemical investigations of Gibson are such as to merit consideration here. Reservations must be made as to the genetic significance of the findings until appropriate analyses are made in the future. Normal human red cells contain about 1% of the hemoglobin as methemoglobin, and it is presumed that an equilibrium exists between these two. Normal functioning hemoglobin predominates because of the action of a reducing system in the erythrocytes.



Methemoglobin

Hemoglobin
Normal O_2 carrier

In normal blood the proportion of the oxidized methemoglobin can be greatly increased by the action of certain oxidizing agents. However, in normal blood, hemoglobin is rapidly regenerated after the removal of the agents that give rise to the oxidation. In contrast to this normal system, individuals with idiopathic methemoglobinemia do not have an active regenerating system for the reduction of methemoglobin to hemoglobin. Herein lies the metabolic disorder. Gibson has provided evidence that the reduction is carried out for the most part by reactions related to glycolysis (see p. 137). The specific reactions shown to be involved are summarized in Fig. 47. The evidence indicates that the flavoprotein carrier is missing or defective in the blood from individuals with idiopathic methemoglobinemia. It is proposed that the reduction can also be effected in the presence of methylene blue as a final carrier in the reduction at the expense of the direct oxidation of glucose and the action of dehydrogenases and coenzyme II (see p. 143). With the abnormal condition *in vivo* the amount of

methemoglobin does not increase indefinitely, and it is suggested that non-specific agents such as ascorbic acid can effect the necessary reduction in a somewhat inefficient manner. There are quite obviously a

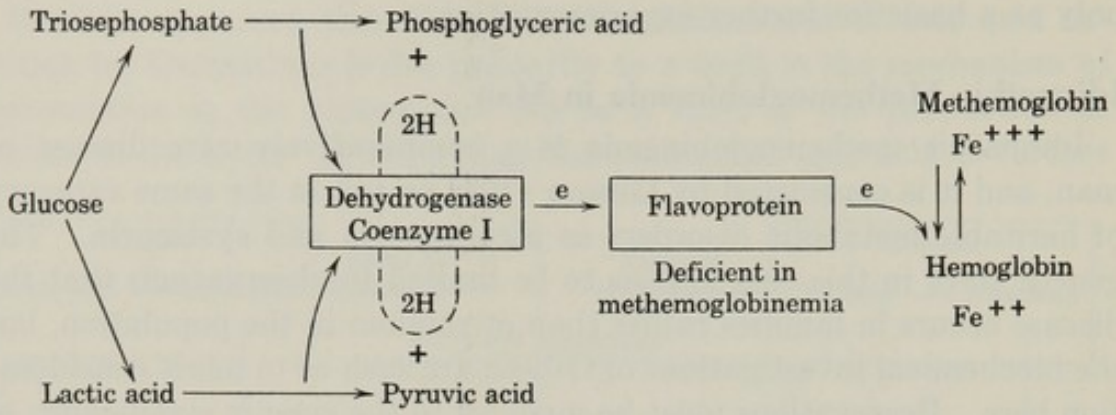


Fig. 47. The reduction of methemoglobin in normal red blood cells.

number of points concerned with this metabolic disorder that require clarification, but there seems to be good evidence that the malfunction has to do with a change in quantity or quality and a resulting activity of a flavoprotein.

Catalase Activity in Higher Plants

The action of catalase on hydrogen peroxide has been discussed previously (p. 146). In higher plants this enzyme is found to be associated largely with the chloroplasts which also contain the chlorophyll of the plant. Von Euler (666, 667) investigated the catalase activity of a number of mutants in barley and found that catalase activity could be correlated with the degree of pigmentation of the plants. That is, normal green barley shoots contain a considerable quantity of catalase activity whereas albino shoots contain less than one-half as much. Thus it appeared that the single dominant gene controlling the presence or absence of chlorophyll also affects the production of catalase. Both catalase and chlorophyll contain porphyrin structures, and an explanation of this phenomenon could rest on an assumption that the mutation concerns porphyrin synthesis. However, the work reported by Eyster (174) seems to provide an explanation for the catalase difference on the basis of degradation of the enzyme rather than on the basis of its formation (Fig. 48). Eyster investigated corn seedlings from albino and yellow mutants as compared to normal green seedlings. Using 1-gram samples of macerated tissue from seedlings grown in a greenhouse (exposed to light) the catalase activities in terms of O_2 in milli-

liters per 5 minutes were found to be: *albino,* 1.53; *yellow,* 2.83; and *green,* 10.14. Seedlings of all three types, when grown in the dark, gave the same value for catalase activity at a level about 25% higher than the green plant grown in the light (12–13). When the dark-grown

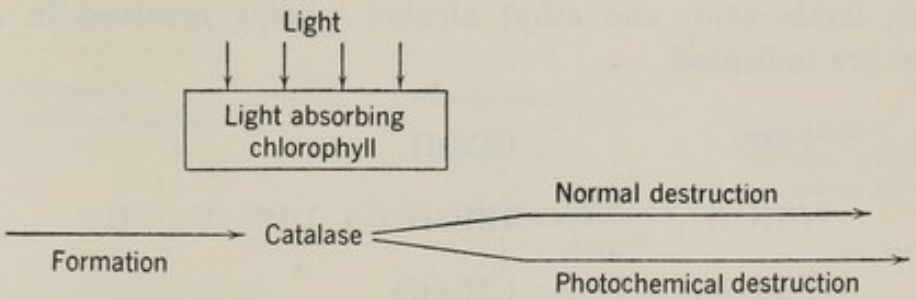


Fig. 48. Catalase in plant seedlings.

seedlings were exposed to light the catalase disappeared at rates in inverse proportion to the green pigmentation. Thus, it seems quite clear that the catalase level is dependent on a balance between the rate of its formation and the rate of its photochemical decomposition. The genetic control of the catalase level is quite indirect on this basis since the chlorophyll apparently merely acts to absorb or reflect destructive radiations and thus protect the catalase.

Flavoprotein and Drug Resistance in Pneumococcus

Sevag and Gotz (559) have investigated the mechanism of resistance of pneumococci to several drugs and have provided some interesting data that may have an important bearing on the present question of enzyme alterations by mutation. Unfortunately the organism used is not known to reproduce sexually, and thus an adequate genetic analysis of apparent heritable change is not possible. A justification for considering the persistent changes in asexual forms as the results of mutations will not be presented here. It will suffice to state that such changes can be produced by the same means as demonstrable mutants in sexual forms and that similar kinds of phenomena result from the treatments; i.e., strains of bacteria having growth requirements for vitamins, amino acids, etc., can be produced by radiation of bacteria just as they can in the sexually reproducing fungus *Neurospora* for example. Still it should be emphasized that much less is known of the mechanism of inheritance in asexual forms than in other organisms.

In the example in question it was found that treatment of pneumococcus cells with each of several drugs, including atabrin, yielded, by single cell isolations, persistent drug-resistant forms. That is, the cells

remain resistant to the drugs through an indefinite number of cell divisions occurring in the absence of the drug. With cells having resistance to atabrin it was found that this resistance is accompanied by changes in the activities of certain enzymatic dehydrogenation reactions. Hydrogen donors used were glucose, hexosediphosphate, glycerol, lactic acid, and ethyl alcohol. Steps involved in such a reaction are indicated.

$$\text{I} \qquad \underset{\text{Glucose}}{\begin{matrix}\text{CHO}\\|\\(\text{CHOH})_4\\|\\\text{CH}_2\text{OH}\end{matrix}} \xrightarrow[\text{Protein}]{\text{DPN}} \underset{\text{Gluconic acid}}{\begin{matrix}\text{COOH}\\|\\(\text{CHOH})_4\\|\\\text{CH}_2\text{OH}\end{matrix}} + \text{DPN}\cdot\text{H} + \text{H}^+$$

$$\text{II} \quad \text{DPN}\cdot\text{H} + \text{H}^+ + \text{Flavoprotein} \rightarrow \text{DPN} + \text{Flavoprotein}\cdot 2e + 2\text{H}^+$$

$$\text{III} \quad \underset{(\text{or } \frac{1}{2}\text{O}_2 + 2\text{H}^+)}{\text{Flavoprotein}\cdot 2e} + \text{Methylene blue} + 2\text{H}^+ \rightarrow$$

$$\text{Flavoprotein} + \underset{(\text{or } \text{H}_2)}{\text{Reduced methylene blue}} \text{ (colorless)}$$

The time required for the decolorization of a given amount of methylene blue was used as an analytical method. It was observed that atabrin-resistant cells show an over-all dehydrogenase activity of about ⅓ to ⅕ that of non-resistant bacteria. Furthermore, it was found that the enzyme preparation from resistant cells is much more susceptible to inhibition by atabrin than is the same system from non-resistant cells. A difference in heat stability of the enzymes was also found. An additional point of consequence is that the addition of riboflavin in relatively high concentrations restores the activity of the atabrin-resistant systems to a normal level in the presence or absence of the drug. The deductions that have been made from a consideration of these facts, and the additional finding that resistant and non-resistant cells contain the same amount of riboflavin, are summarized in Fig. 49. It will be noted that the summation of experimental facts points to the production of a protein with an altered specificity by the resistant bacteria. The atabrin resistance is a relative one, and the reduced dehydrogenase is still not a markedly limiting factor in growth of resistant strains.

The above example is an especially interesting one in that the data indicate a change in protein specificity resulting from a probable mutation. It is true that this system has not been isolated and compared

with the normal one, but there has been a sufficient amount of work on this kind of system to provide an excellent background for carrying out such experiments. On the genetic side of the question it may be

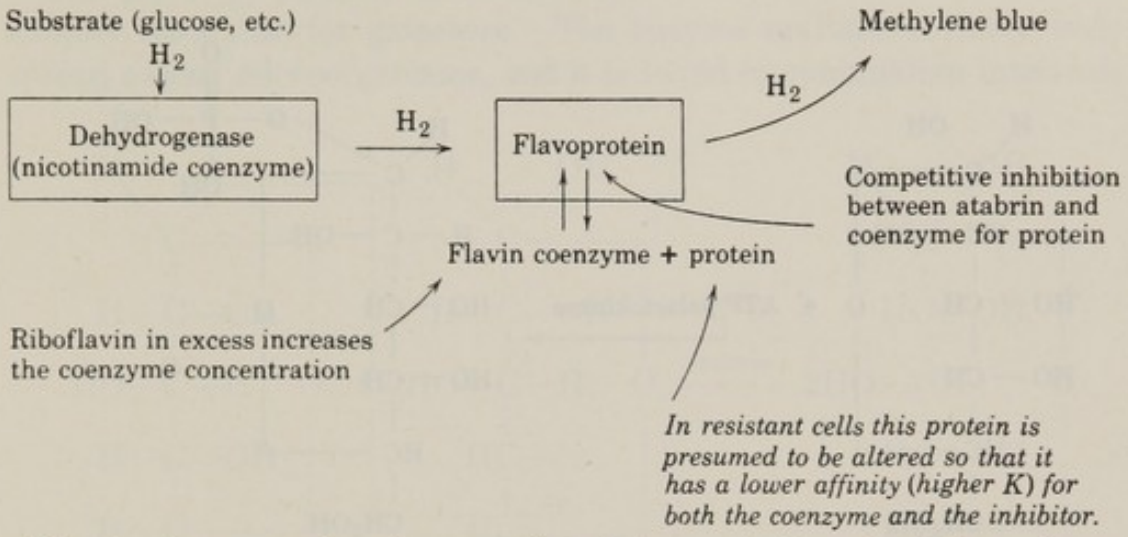


Fig. 49. Summary of an interpretation of the mechanism of atabrin resistance in *Pneumococcus*.

suggested that atabrin-resistant mutants with the same properties might be obtained in a similar way in organisms that permit a genetic analysis.

Fermentation Enzymes

A very large amount of work has been done on the inheritance of capacities for fermentation of a variety of carbohydrates. For the most part these experiments were carried out with living cells and in connection with the potentialities for adaptation to fermentative action with yeast, bacteria, and fungi which often show the capacity to utilize certain sugars only after a period of standing in the presence of the substrate. It is quite clear that this potentiality for fermentations is inherited just as is an immediate fermentive capacity or the lack of fermentive capacity (see Chapter 11). In most cases these phenomena have not been analyzed on the basis of isolated enzyme systems, and thus it is not clear just what biochemical reactions are inherited.

It may be presumed that the heritable fermentations systems in yeast such as those for lactose, raffinose, maltose and galactose proceed through conversion to a normally fermentable sugar such as glucose which in its turn is broken down by the usual pathways (see p. 137). When this is true the reactions dependent on heritable characters are those concerned with the conversion of the substrate to one which is

used in a normal system. For example, it has been shown that yeasts able to ferment galactose can do so by the system shown in Fig. 50 (89). Thus the conversion of galactose into a substrate suitable for the glycolysis system involves two steps: a phosphorylation by a hexo-

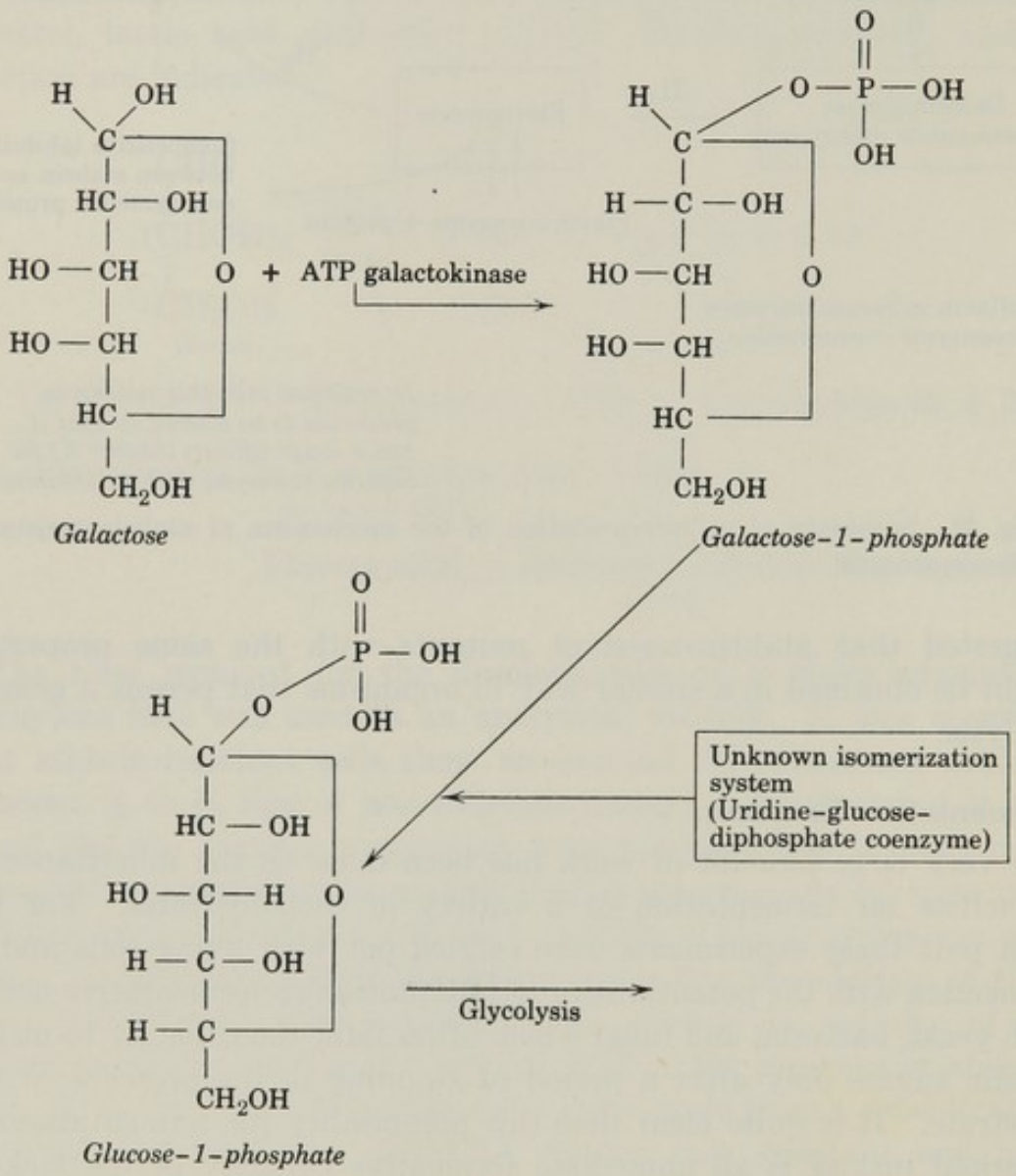
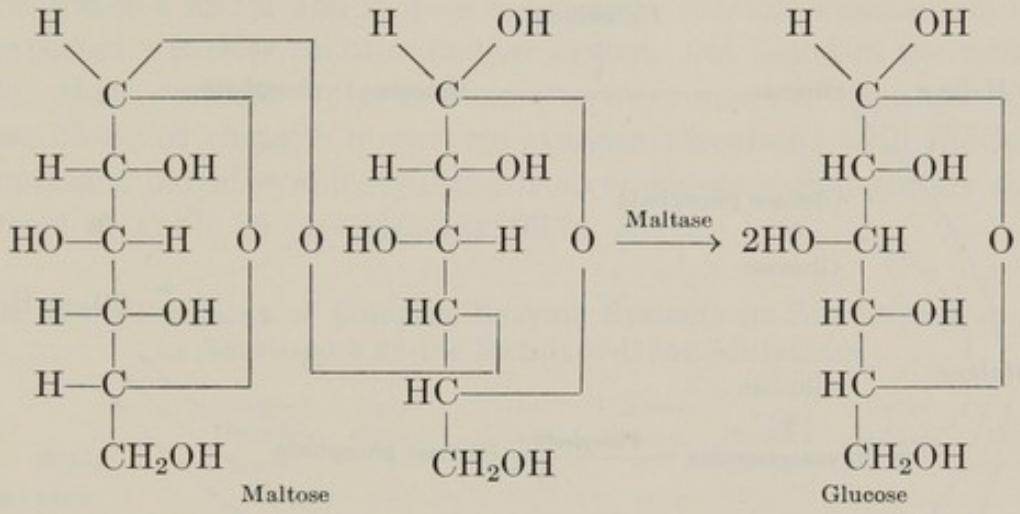


Fig. 50. The isomerization of galactose to glucose.

kinase that is different from that acting on glucose, mannose, or fructose; and an inversion of the spatial configuration around the fourth carbon atom of the galactose. The galactose hexokinase has been purified and the first step has thus been separated from the second, but the mechanism and the reacting components of the latter reaction are not well known. Presumably another enzyme with uridine-diphosphate-glucose as a coenzyme is involved. Evidence has not been pre-

sented as to what part of this system is non-operative in yeast strains that do not ferment galactose. This system of course may not be the only one involved in the process.

The primary reaction in maltose fermentation could be somewhat simpler than that for galactose. The enzyme maltase is fairly widespread among microorganisms, and it is found in mammalian intestines.



Thus it might appear that the genetic control of maltose fermentation concerns the production of maltase. On the other hand an enzyme producing a phosphorylitic cleavage could be involved. Actually there is evidence that the problem may be much more complex than suggested above. Monod and Torriani (436) and Doudoroff et al. (149) investigated mutants of *E. coli* that are able to ferment maltose rapidly but glucose only slowly. It was demonstrated that both the mutant and the parent strain (which ferments maltose and glucose equally rapidly) contain an enzyme, "amylomaltase," that converts maltose to a polysaccharide of a size sufficient to give a blue color with iodine. The polysaccharide can then be broken down to give glucose-1-phosphate, and frustose-6-phosphate. The difference between the mutant and wild type is not explained by this roundabout mechanism of the metabolism of maltose. (It should be noted that amylomaltase catalyzes reversible reactions in the exchange of groups between polysaccharides of various sizes without involving the formation of phosphate esters.)

Another kind of reaction of maltose has been investigated by Stodola and Lockwood (612). They observed that certain species of *Pseudomonas* oxidize maltose to maltobionic acid. The free aldehyde group of maltose is converted to a carboxyl without rupture of the glucosidic

linkage. The importance of this kind of metabolism in other organisms such as yeast is not known. In any case, it is appropriate to set up a general description of some of the possibilities that will illustrate the biochemical problem encountered in investigating inheritance of maltose-fermenting capacities. As shown in Fig. 51, possibilities of multiple interrelated and simultaneous pathways exist.

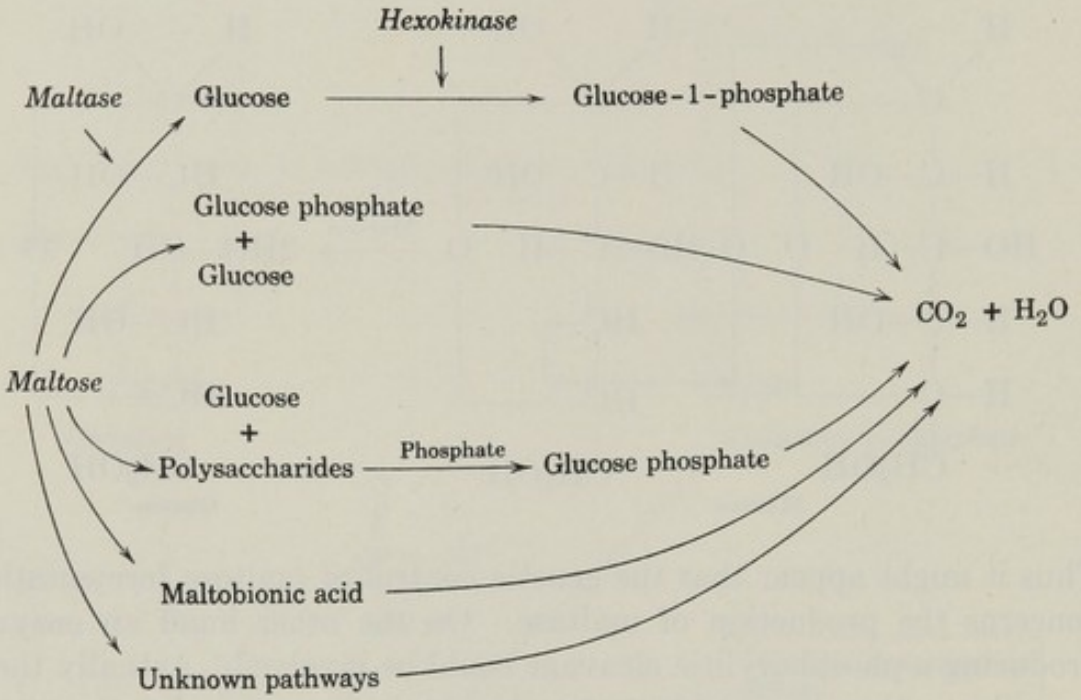


Fig. 51. Some possible pathways for maltose fermentation.

Unfortunately there are relatively few known examples in which apparent single-gene mutations result in changes of enzyme complexes such as those carried in mitochondria or microsomes, although a great deal of interest has been centered on such units in connection with cytoplasmic inheritance (see Chapter 12). Ephrussi (170, 172) and associates have described a strain of yeast (petites segregationals) in which a single-gene mutation results in a loss, within the limits of sensitivity of the analytical methods used, of several components of the succinoxidase system. These include succinic dehydrogenase, cytochrome *b*, and cytochrome *a*. Cytochrome *c* is present in nearly a two-fold excess. Such yeast strains respire more or less normally in the absence of oxygen, but in the presence of oxygen they utilize glucose at less than 10% of the normal rate. Such a result would be expected from the independent observations on the deficiencies in components of the succinoxidase system. Two somewhat analogous gene mutant strains of *Neurospora* have been described (429). One of these (strain

C115) contains an excess of cytochrome *c* in the large cytoplasmic particles (mitochondria), an abnormally low level of cytochrome *b*, and little or no cytochrome *a*. The second strain (C117) contains large particles having a great excess of a heme component with the absorption spectrum of cytochrome *b*. A substance with properties similar to those of cytochrome *e* (654) is also present. This component is not observed in wild-type *Neurospora*. The mutant is deficient in cytochromes *c* and *a* and it does not possess succinic oxidase activity as expected. It does contain another system, not found in the normal mold, that destroys cytochromes ("cytochromase") (see p. 338). These observed changes in enzyme systems associated with the large cytoplasmic particles and resulting from single-gene changes are summarized in Table 26 (see also Fig. 99, p. 339).

Table 26. Alterations of Complex Enzyme Systems in *Saccharomyces* and in *Neurospora* as the Result of Gene Mutations

Strain	Succinic Dehydrogenase	Cytochrome *b*	Cytochrome *c*	Cytochrome *a*	Succinic Oxidase	Cytochrome *e*	Cytochromase
Normal yeast	+	+	+	+	+	−	?
"Petite" yeast segregational	−	−	++	−	−	−	?
Wild-type *Neurospora*	+	+	+	+	+	−	−
Neurospora C115	+	+	++	−?	+?	−	?
Neurospora C117	+	++	−	−	−	++	+

It is clear from these results that mutation of single genes can cause deep-seated alterations in whole enzyme complexes, and it is not possible to correlate a presumed gene function with the formation of any one enzyme. Indeed, the changes in large particles that are listed are only those that have been observed, and it remains to be established what other enzyme functions are altered in these systems. It is already known that the soluble enzyme fraction of "petite" strains of yeast contain double the normal activity of lactic dehydrogenase (568) and that both particles and soluble enzymes of the *Neurospora* mutants carry an abnormally high quantity of flavin-adenine-dinucleotide presumably associated with the activities of specific enzymes. These examples of the results of gene changes on the constitution and activities of enzyme complexes are not necessarily more complicated than the cases in which there appears to be a simple relation between gene function and the formation of a single enzyme. Multiple changes have not usually been looked for in the presumedly simpler examples.

4. Some General Conclusions

There is no doubt whatever that mutations result in the alterations of cellular capacities for carrying out biochemical reactions. It is also quite clear that many of these alterations that have been observed are due to changes in quantity, quality, or activity of enzymes, enzyme complexes, or other macromolecular systems that undergo specific kinds of chemical combinations and thus cause non-random orientations of molecules. The question still remains open as to whether these units or systems are direct products of gene action. They are themselves metabolites each subject to chemical changes under the influence of various non-genic catalysts that exist in living cells. Since this is true, a direct gene action need not be essential for the formation or destruction of a specific protein, nucleic acid, or polysaccharide. For example, when it can be shown that a cell or tissue contains more than one molecular species of a protein with a similar but not identical structure or catalytic function it is reasonable to assume that the similar molecules are produced, one from another, by enzyme action. All the examples of gene enzyme relations that have been described here can be explained on this basis.

On the other hand, it has been a fairly generally accepted view that enzymes and other macromolecules have their specificities determined by direct gene action in which the genes participate by serving as models or templates. No direct experimental evidence supports this view, but all the examples of gene-enzyme relations given here can also be described on this basis as well as on the indirect one already discussed. It is reasonable to conclude that these extreme points of view merely suggest the limits of the possible mechanisms of gene action. It is not at all obvious that all genes must act in the same fashion, and each of the enzymes or other specific macromolecules that are known at present can be a direct or a very indirect product of a functioning gene. Perhaps the action of a gene is best described in the same fashion as any other agent that influences the course of a biochemical reaction (see Fig. 65, p. 219). That is, it can serve as a catalyst or an inhibitor in the production or destruction of some cellular component, and the primary action lies in its influence on the balance of reaction rates.

CHAPTER 8

Metabolic Patterns

Since genes exert their effects through control of metabolism, the attainment of a real understanding of inheritance is dependent not only on knowledge of the existence of these heritable units and the mechanisms of their transmission, but it is also dependent on a complete knowledge of cellular biochemistry. That is, it is just as necessary to know exactly what is being controlled as it is to know what exerts the control and how it comes about. This purely biochemical problem has received a great deal of attention quite independently of its interest to genetics, and we now know many details about a great number of chemical reactions that occur in organisms. The majority of these reactions have been studied in isolated systems of varying degrees of complexity as, for example, with highly purified single enzymes or mixtures of purified enzymes. These methods give information on what reactions may potentially occur at reasonable rates in a tissue and as such they are highly significant.

It is essential to note that, even though a whole consecutive series of reactions can be described in terms of known substrates and purified single enzymes, it is not necessarily a fact that this reaction series is important or even that it goes on significantly *in vivo*. Experimental methods in biochemistry that have made use of isolated cellular units such as nuclei or mitochondria, or procedures using tissue homogenates, or tissue slices, no doubt have given information on biochemical potentialities that are more nearly like those of intact living tissues. Even these techniques, however, do not give a complete picture, and results are often difficult to interpret. Thus, as biochemical systems that are amenable to experimental work become more nearly like intact living cells, possibilities of interactions between the numerous individual reactions that are going on increase enormously. In addition, it is clear that these interactions can be increased or decreased because of intra-

cellular organization. That is, if two potentially interacting systems (perhaps interacting through a common substrate, coenzyme or enzyme) are included in the same cellular particle, one might expect a different effect than if the systems are included in different particles or locations in an intact cell.

Thus, the genetic control of metabolism is no less complex than metabolism itself. An understanding of the problem must include knowledge of the effects of cellular organization and the concept of the dynamic state of metabolism, as well as information on the metabolic potentialities of the organism in terms of single biochemical reactions. It is for these reasons that the emphasis here, so far as the effects of mutations are concerned, has been placed on the alterations of reaction rates rather than on presence or absence of absolute capacities for carrying out specific biochemical reactions. It is perfectly conceivable that a change in a reaction velocity of only a few percent can make the difference between black or white coat color in a mammal, or even between the life or death of an organism. It must, of course, be recognized also that a change in the rate of one reaction necessitates a reshuffling of rates of interdependent reactions so that the final expression of the change may appear simple and actually be very complex indeed because of this shift in the existing metabolic pattern.

Most of the above discussion has been considered in more detail in earlier chapters, but these points are repeated here because they should be kept in mind during a consideration of the contributions made by studies of mutants to knowledge of metabolic patterns. As described in Chapter 5, the principles involved in this approach are quite simple. It is considered that a mutation causes the loss of capacity for carrying out some specific biochemical function. When this change is lethal because of the failure of some step in a biosynthesis of an essential metabolite, then addition of this metabolite as a nutritional supplement may alleviate the effect of the mutation and the organism survives. As described previously (p. 108), whole reaction series can be studied in this way, using a series of different mutants that have requirements for the same metabolite. When this procedure works it is indeed a valuable technique and, as will be shown, it has made many significant contributions to knowledge of the nature of metabolic patterns. There are, however, certain limitations and complications that should be considered. As noted in Chapter 5 (p. 104), direct experiments have shown that the class of nutritional mutants used for investigating metabolic patterns in *Neurospora* represents not more than one-tenth of the mutants present after inducing mutations. This is cer-

tainly a maximum figure since growth was the criterion used for mutant selection. For this reason alone it is not to be expected that all important metabolic reactions or reaction series of the organism are represented among the mutants commonly obtained.

Similar selections no doubt occur in the processes of isolation of mutants of other organisms. There are many good reasons why selection of this kind would be expected even in the absence of direct experimental evidence. The experimental procedure depends on mutants selected having the capacity to utilize an essential metabolite supplied from the environment. Thus, all mutants with requirements for large or small molecules that will not pass through cell membranes would be eliminated. In addition, strains that carry mutations that cause a disruption of essential cellular organization to an excessive degree would not be found. Mutants of yeast (169), higher plants (154), and *Neurospora* (429) that do have aberrations in organized systems have been described. These grow, but slowly under appropriate conditions, and they probably represent examples of relatively mild disorganizing effects of mutation. It is also to be expected that mutations affecting systems in dynamic equilibrium where rapid turnover or production of energy by the interconversion of a large number of compounds is an essential feature, may give rise to strains which cannot survive regardless of the environment.

Another important property of the mutants used for investigations of metabolic patterns is concerned with partial genetic blocks in biochemical reactions. Many mutants that show partial blocks in an obvious fashion have no doubt been discarded by most investigators since the phenotypes of such strains are not always clear-cut, and they are frequently variable due to the action of ever present modifying genes. It is not necessary, however, to bring in mutants that have not been considered deficient enough to work with in order to demonstrate clearly the phenomenon of partial genetic blocks. A very convincing example was provided by an early investigation of a riboflavin mutant of *Neurospora*. This strain was shown to be able to grow without riboflavin at 25°C, but at 35°C it would not grow at all in the absence of the vitamin (p. 293). A partial requirement could be demonstrated at intermediate temperatures. The common occurrence of such temperature-sensitive mutants is, in itself, adequate evidence for partial genetic blocks, but even more striking evidence was obtained from studies of the behavior of the riboflavin mutant at 34°C. At this temperature no growth was obtained without added flavin, but it was shown that when a very small amount of the vitamin is supplied growth

and riboflavin synthesis occurs alternately as illustrated in Fig. 52. Other examples, with and without temperature sensitivity, were shown to exist among the adenine (420) and the pyrimidine (421) mutants

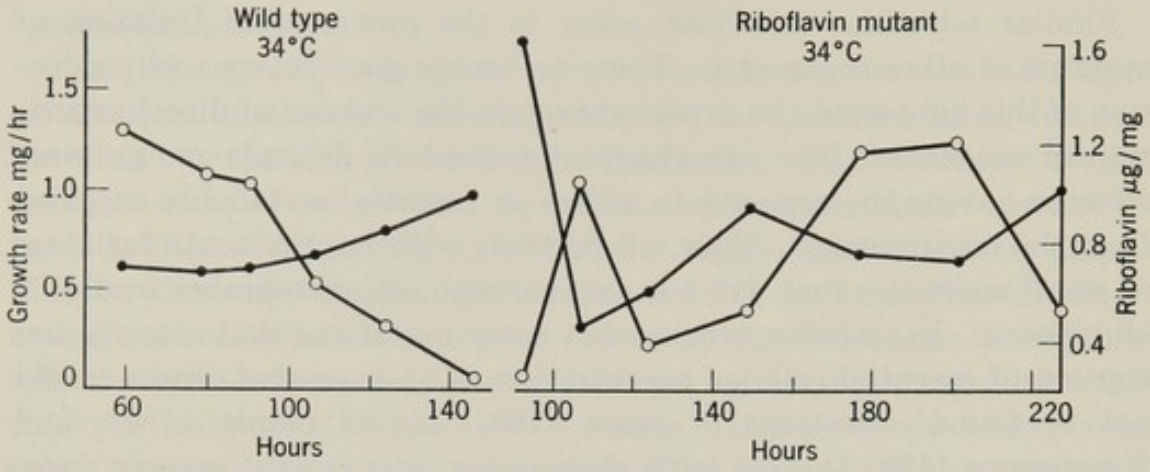


Fig. 52. Riboflavin production by wild-type *Neurospora* and a riboflavin-requiring mutant. Growth rate (scale on left) is indicated by the open circles; the riboflavin content is shown by the solid circles (scale on right). Cultures were grown in the presence of a limiting concentration of riboflavin (0.015 µg per ml of culture medium). After Mitchell and Houlahan (419).

of *Neurospora*. Further work, also with *Neurospora* mutants, by Bonner and collaborators (57) has made use of isotope tracers in a clear demonstration of partial genetic blocks in some tryptophan

Table 27. Partial Blocks in Tryptophan-Requiring Mutants of *Neurospora* as Demonstrated by the Use of N^{15}-Labeled Compounds *

Strain Number	N^{15}-Labeled Compound Supplied	Compound Isolated	N^{15} Content of Isolated Compounds Given as % of Total Amount Isolated
39401	Anthranilic acid	Acetyltryptophan	67
7655	Anthranilic acid	Acetyltryptophan	37
		Quinolinic acid	40
10575	Indol	Acetyltryptophan	80
		Quinolinic acid	75
C83	Tryptophan	Acetyltryptophan	98.9
		Quinolinic acid	94.3

* From Bonner, Yanofsky, and Partridge (57).

mutants. Some data are summarized in Table 27. (See Fig. 53 for a description of a metabolic pattern involving tryptophan.) Particular note should be made of the results with mutant C83 which provide the

most convincing evidence yet reported that mutation to a complete block of an essential reaction can occur without an entirely lethal effect. But even this example does not constitute proof of complete absence of tryptophan synthesis (assuming the deviation from 100% as not significant) since, in the experiments conducted, the tryptophan precursors that might cause dilution in the labeled compounds isolated may simply have been used up in the side reactions too rapidly to leave enough for a significant conversion to tryptophan by an inefficient system. Direct evidence based on enzyme studies which show that tryptophan synthesis is inefficient at best in strain C83 has been discussed earlier (p. 168, Chapter 8). These experimental facts, as well as a good deal of less direct evidence that could be cited, require consideration in interpretations of experimental results obtained from studies of mutants for the purpose of establishing the nature of metabolic patterns. For example, a nutritional mutant with a partial block may grow in the presence of only one metabolite if the partial block permits sufficient synthesis of another that would also be required if the block were complete. A case in point is *Neurospora* strain 39401 (Table 27) which will synthesize an adequate amount of tryptophan while growing in the presence of a much smaller quantity of niacin.

Before proceeding to a discussion of the metabolic patterns that have been devised through studies of mutants, attention should be directed toward the question of what information is required in order to establish whether a particular compound is actually an intermediate in the biosynthesis of some other metabolite. There is no simple answer to this question. Many of the substances that we consider to be important nutrients may never exist in the free state in cells that synthesize them. For example, mutants that require riboflavin may use the vitamin from external sources only by virtue of having a phosphorylating system that can convert the compound to the functional coenzyme form. The biosynthesis of the flavin coenzymes may well proceed by formation of the isoalloxazine ring system on a polyhydroxy compound that is already phosphorylated, and thus riboflavin itself is not necessarily a normal precursor to coenzymes that contain it. The same principle may apply in many biosyntheses and indeed there is direct evidence that it does, as will be discussed in connection with the formation of histidine and nucleic acid components.

It has been considered that when a compound is accumulated by one mutant of a series and utilized by another mutant of the same series, the substance can be considered an intermediate. This is a useful and practical point of view, but such evidence does not establish

the point. It may mean only that the compound can be produced from and reconverted to a true intermediate, not necessarily even by the same pathway. Experiments with isolated enzymes can provide useful contributing evidence. That is, if compound A is converted to compound B through catalysis by a single enzyme, and B is converted to C by a different single enzyme, then B can be considered an intermediate in the conversion of A to C. Unfortunately, this approach is often not practicable, especially when dealing with organized enzyme systems where individual enzyme activities cannot be separated. In such cases it is not unlikely that the intermediates in biosynthesis are actually enzyme-substrate complexes so that separation of activities would give a misleading result in any case.

Manifestly other problems complicate the process of establishing metabolic patterns through studies of mutants, and at least some of these are pointed out in the more specific discussions that follow. The patterns that are presented represent gross over-all pictures, lacking in details, for the most part, and rarely taking into account the nature of more than one reactant or product in each step of a reaction series. That is, attention is usually focused on the biochemical changes that occur in the formation or degradation of some principal carbon chain or other structural unit. It is convenient to do this for descriptive purposes, but one must guard against the illusion of simplicity that is so created.

A good deal of information has now been obtained, largely from studies of nutritional mutants of microorganisms, that gives evidence on the pathways of biosynthesis of the amino acids, the heterocyclic components of the nucleic acids, the vitamins, the fatty acids, and some miscellaneous metabolites. Even the simple carbohydrates are notably absent from this list as are the polysaccharides, the proteins, the nucleic acids, and the more complex lipids.

1. Amino Acids

Aromatic Amino Acids and Related Compounds

Investigations of mutants concerned with the biological synthesis and degradation of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan have made possible a description of an extensive and complex metabolic pattern (Fig. 53). A variety of organisms have been studied, including vertebrates, insects, higher plants, fungi, and bacteria. The entire pattern shown in the figure is not known to occur

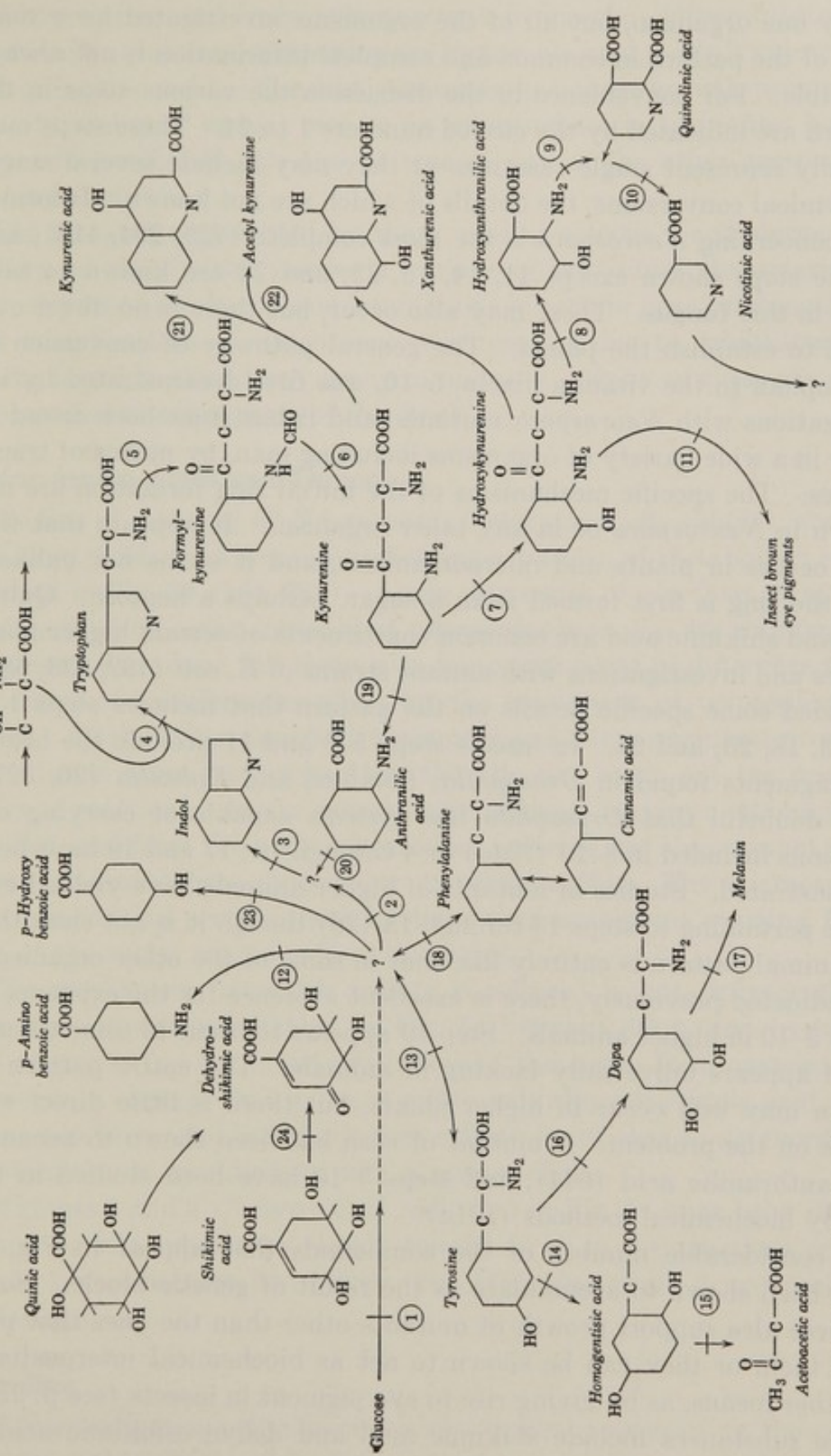


Fig. 53. Biosynthesis of the aromatic amino acids. Known positions of genetic interference in reaction series are indicated by the circled numbers on the cross bars. (See text for full explanation.)

in any one organism, but all of the organisms investigated have some parts of the pattern in common and complete information is not always available. For convenience in the discussion the various steps in the pattern are indicated by the circled numbers 1 to 24. These steps may actually represent single reactions or they may include several stages of chemical conversions, the details of which are not known. Information concerning *Neurospora* is the most complete (125, 294, 116), and all the steps shown except 11, 14, 15, 23, and 24 are known to take place in this fungus. These may also occur, but there is no direct evidence to establish the points. The general pathway of conversion of tryptophan to the vitamin niacin, 5–10, was first demonstrated by investigations with *Neurospora* mutants, and it has since been found to occur in a wide variety of organisms including man, by means of tracer studies. The specific mechanisms of the initial ring formation are not known in *Neurospora* or in any other organism. It appears that this only occurs in plants and microorganisms, and it seems not unlikely that the ring is first formed from a sugar, perhaps a heptose. Quinic acid and shikimic acid are common constituents of certain higher plant tissues and investigations with mutant strains of *E. coli* (132, 133, 684) provided some specific details on the pattern that includes steps 1–4, 12, 13, 18, 23, and 24. In insects steps 5–7 and 11 provide the brown eye pigments found in *Drosophila, Bombyx,* and *Ephestia* (26, 327). It is doubtful that *Drosophila* has systems capable of carrying out reactions included in 8–10 (704*a*) or 4 through 16; 17 and 19 have been demonstrated. Studies of mutants of higher animals have yielded evidence pertaining to steps 13 through 18 (26) though it is not clear that the animal pattern is entirely like that in some of the other organisms. As indicated previously, there is excellent evidence for the existence of steps 5–10 in higher animals. Step 19 appears to occur in some tissues, but 4 appears universally lacking in animals. The entire pattern as shown may well occur in higher plants, but there is little direct evidence on the problem. A mutant of corn has been shown to accumulate anthranilic acid (644), and steps 5–10 have been studied in the pea by biochemical methods (191*a*).

A considerable number of the compounds that appear in Fig. 53 have been shown to accumulate as the result of genetic blocks. Some of these also support growth of mutants other than the ones that produce them or they can be shown to act as biochemical intermediates by other means, as by giving rise to eye pigment in insects (see p. 214). These substances include shikimic acid and dehydroshikimic acid in *E. coli,* anthranilic acid, and hydroxyanthranilic acid in *Neurospora,*

and kynurenine and hydroxykynurenine in insects. Other substances produced in abnormal quantities are kynurenic acid, acetylkynurenine, and quinolinic acid. The last substance has some growth-promoting activity and may or may not be an intermediate in the series, but as discussed previously this can be true of any of the other compounds accumulated.

The mutants represented by those used as a basis for studying this pattern of metabolism possess a variety of individual characteristics such as partial blocks, unique interactions in certain gene combinations, and complications of nutritional requirements due to inhibitions, as can be found in almost any group of mutants. Many of these are used as examples in other parts of this book.

Sulfur Amino Acids and Threonine

Studies of mutants of *Neurospora, Ophiostoma, B. subtilis,* and *E. coli* have yielded information indicating a pattern of metabolism summarized in Fig. 54 (164, 289). Again this series of reactions, that can be described by using mutants of microorganisms, has its counterpart in mammalian tissue, but there is an important point of difference from the series of reactions described for the conversion of tryptophan to niacin. In the latter case both mammals and microorganisms produce the vitamin from tryptophan but they do not carry out the reverse series of reactions. In the present case animals require methionine in the diet, and from this they can produce cysteine but they cannot produce methionine from cysteine or inorganic sulfur. The microorganisms are able to produce both cysteine and methionine starting from any of the sulfur compounds in the series.

The mechanism by which sulfate is reduced is not entirely clear. The three mutants indicated in Fig. 54 between $SO_4^=$ and cysteine, will of course use the other substances of the series from cysteine on. However, a mutant that will use sulfite will also use cysteic acid, and it has not been established whether sulfate is reduced as an inorganic substance or in combination with a carbon chain.

Mutants 1 and 2 (*Neurospora*) indicated in Fig. 54 have been shown to accumulate substances closely related to or in the reactions series. Strain 1 accumulates homoserine and threonine while 2 accumulates cystathionine.

Histidine

The investigations that have yielded evidence concerning the pathway for biosynthesis of histidine illustrate a number of the points

considered in the introduction and general discussion in this chapter. The various mutants used accumulate a variety of compounds related in chemical structures to histidine, some of them accumulate unrelated

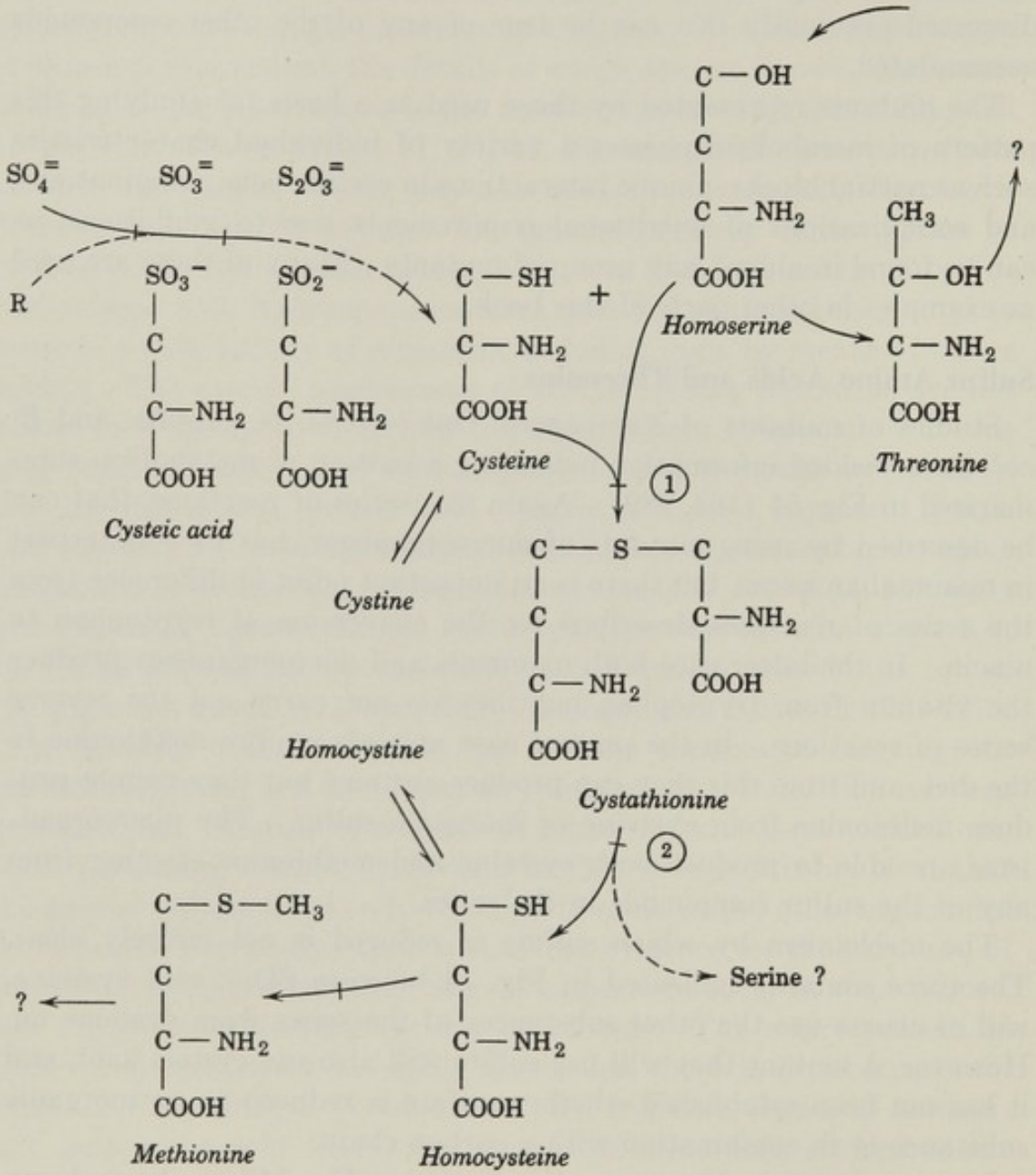


Fig. 54. Biosynthesis of the sulfur amino acids and threonine. The cross bars indicate positions of known genetic blocks.

compounds, pyruvic acid and α-ketoisovaleric acid, and all of the *Neurospora* histidine mutants are subject to a potent and complex inhibition by certain combinations of amino acids other than histidine. The pattern of biosynthesis so far as it is known is summarized in Fig. 55.

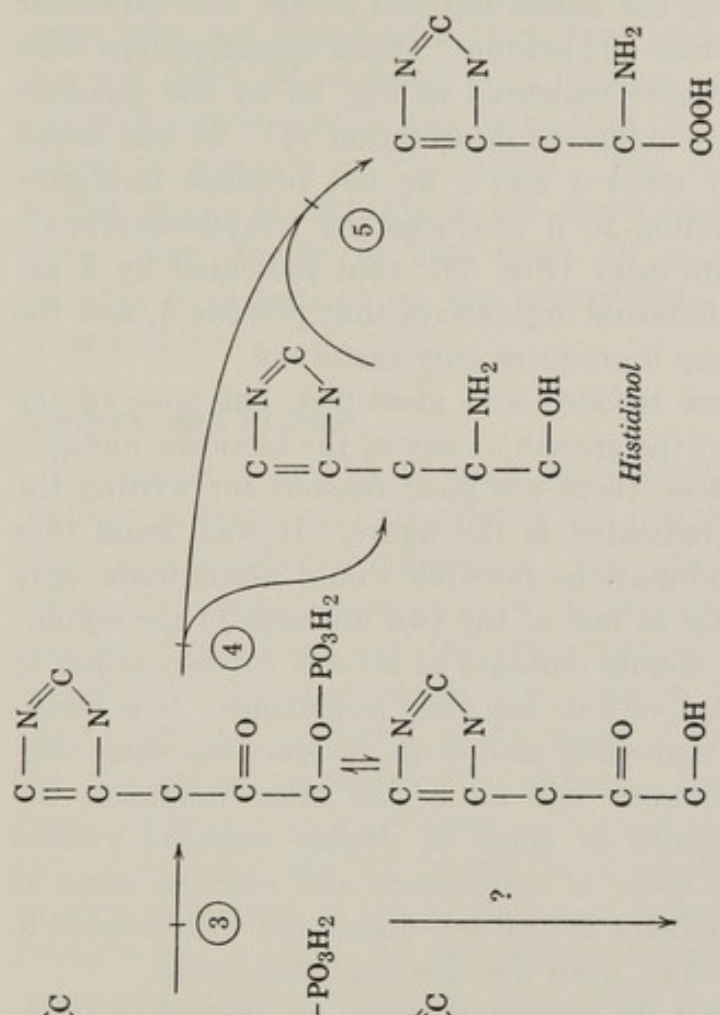


Fig. 55. The biosynthesis and degradation of histidine. Circled numbers indicate different mutant types known in *Neurospora* or *E. coli*.

It was first found (664) that a mutant strain of *E. coli* will accumulate sizable quantities of the amino alcohol, histidinol. It was also found that another strain of the bacterium will utilize this substance with fair efficiency in the place of histidine. Other investigations with a series of *Neurospora* mutants indicated in Fig. 55 by the numbers for steps 1–5, provided more extensive information (8). It was found that mutants indicated by steps 1 and 2 do not produce imidazole derivatives, that corresponding to 3 produces the trihydroxypropylimidazole and its phosphate ester (Fig. 55), that indicated by 4 accumulates all five of the imidazole derivatives that precede 4, and the mutant corresponding to step 5 produces only histidinol.

All these compounds were isolated and identified, but none of the pure substances will support the growth of any of the histidine mutants of *Neurospora*. Nevertheless, there are good reasons for writing the compounds in a series as indicated in the figure. It was found that double mutants in all combinations possible would accumulate only the imidazoles characteristic of one of the two mutants in the combination. For example, the double mutants of strains corresponding to steps 1 or 2 with those of 3, 4, or 5 do not yield imidazoles. It is therefore assumed that 1 and 2 come first in a series of reactions since they block the formation of histidine and all of the other imidazole derivatives. Other combinations of genes in double mutants yielded results consistent with the order of compounds and reaction steps as shown in the figure. The scheme is also self-consistent with respect to a reasonable series of reactions from the chemical standpoint.

It has been postulated that the phosphate esters are the actual intermediates in the series and that these are not used for growth because the cell membranes of the mold are not permeable to such esters. This is consistent with other known facts. The non-phosphorylated derivatives are presumably not used because of the absence of the proper phosphorylating enzymes in the fungus, but it appears that *E. coli* may have a system that will phosphorylate histidinol at least.

The histidine mutants of *Neurospora* were so classed because they all require histidine for growth, but it is an equally inherent character that these strains are all inhibited by particular combinations of other amino acids. For example, the requirement for histidine is increased many-fold if the mutants are cultured in a medium containing arginine plus tyrosine. Other basic amino acids such as lysine or ornithine have the same effect as arginine, and tyrosine can be replaced by a variety of other neutral amino acids. Significant inhibitions occur only with combinations of at least two amino acids, one basic and one

neutral, and since most protein hydrolysates contain an insufficient amount of histidine to counteract the inhibitory action of the other components of such mixtures, histidine-requiring mutants of *Neurospora* were not found until they were looked for specifically. When this was done, by selecting on a medium containing only histidine as a growth supplement, it was found that this is one of the more common classes of mutants in this fungus. The mechanism of the inhibition in this group of mutants is not understood, but the results demonstrate very clearly to how great an extent selection in obtaining mutants is dependent on the experimental conditions chosen by an investigator.

Arginine and Proline

As indicated in Fig. 56 arginine, proline, ornithine, citrulline, and perhaps glutamic acid are related in a metabolic pattern that probably exists in a number of organisms. The ornithine cycle was well established for animal tissues prior to the application of mutants in investigations of this kind, and the demonstration, by means of *Neurospora* mutants, of the existence of this cycle in the fungus (591) provided one of the first examples of the value of the use of genetic blocks in the elucidation of metabolic patterns. Evidence that this series of reactions begins with glutamic acid is not entirely clear. If this is the case, it appears that the formation of ornithine from glutamic acid is entirely analogous to the formation of lysine from α-aminoadipic acid. Perhaps this is a basis for the complex metabolic interrelations between lysine and arginine that have frequently been observed in microorganisms. It is even possible that the same enzyme operates in both series.

The ornithine cycle has been shown to exist in *Lactobacillus* by use of a series of natural strains or spontaneous mutants of the organism (665). In the fungus, *Aspergillus,* a mutant has been found that will utilize for growth proline, ornithine, or arginine but not citrulline (490), a fact suggesting that, in this organism, citrulline is not an intermediate in the reaction series. It is quite possible, however, that this is a phenomenon of gene interaction as has been found in *Neurospora* (see p. 269). In this organism it has been shown that the apparent position of a genetic block in this series as well as the quantitive requirement can be altered a great deal by introducing other mutant genes.

Growth of all of the arginine mutants is inhibited by lysine in *Neurospora,* but conditions were not such as to eliminate this group during mutant selection as they were with the histidine mutants.

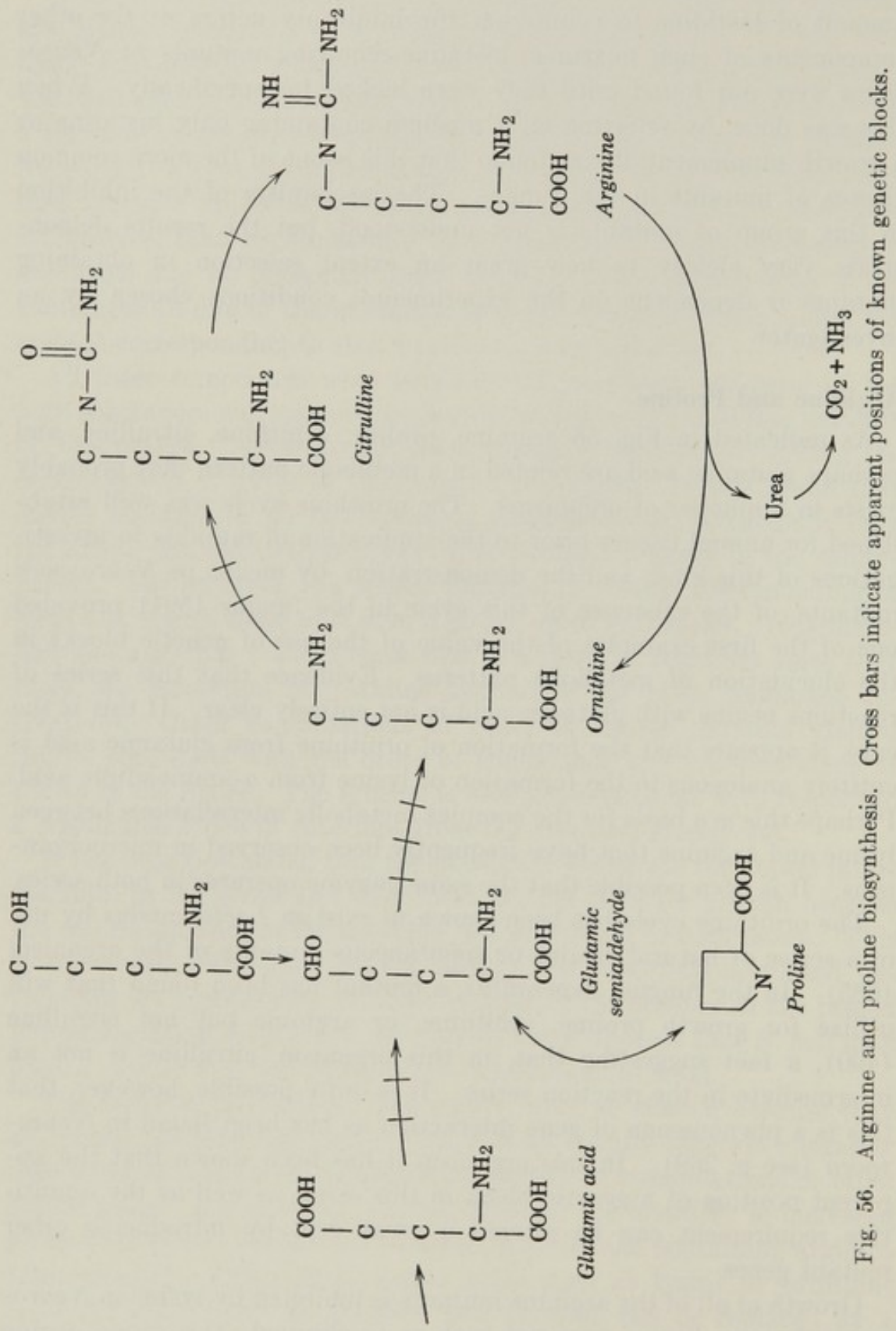


Fig. 56. Arginine and proline biosynthesis. Cross bars indicate apparent positions of known genetic blocks.

Lysine

Mutants having nutritional requirements for lysine have been found commonly in a variety of organisms, and evidence has been obtained concerning pathways of biosynthesis of this amino acid. However, in contrast to some of the other patterns that have been described, here there is good reason to believe that lysine can be formed by two completely different pathways in two different organisms. In connection with the metabolic pattern concerned with the biosynthesis of the aromatic amino acids (p. 194) it is pointed out that the entire pattern is not found in all organisms but that the portions of it that overlap are very similar in different species. As shown in Fig. 57 this does not

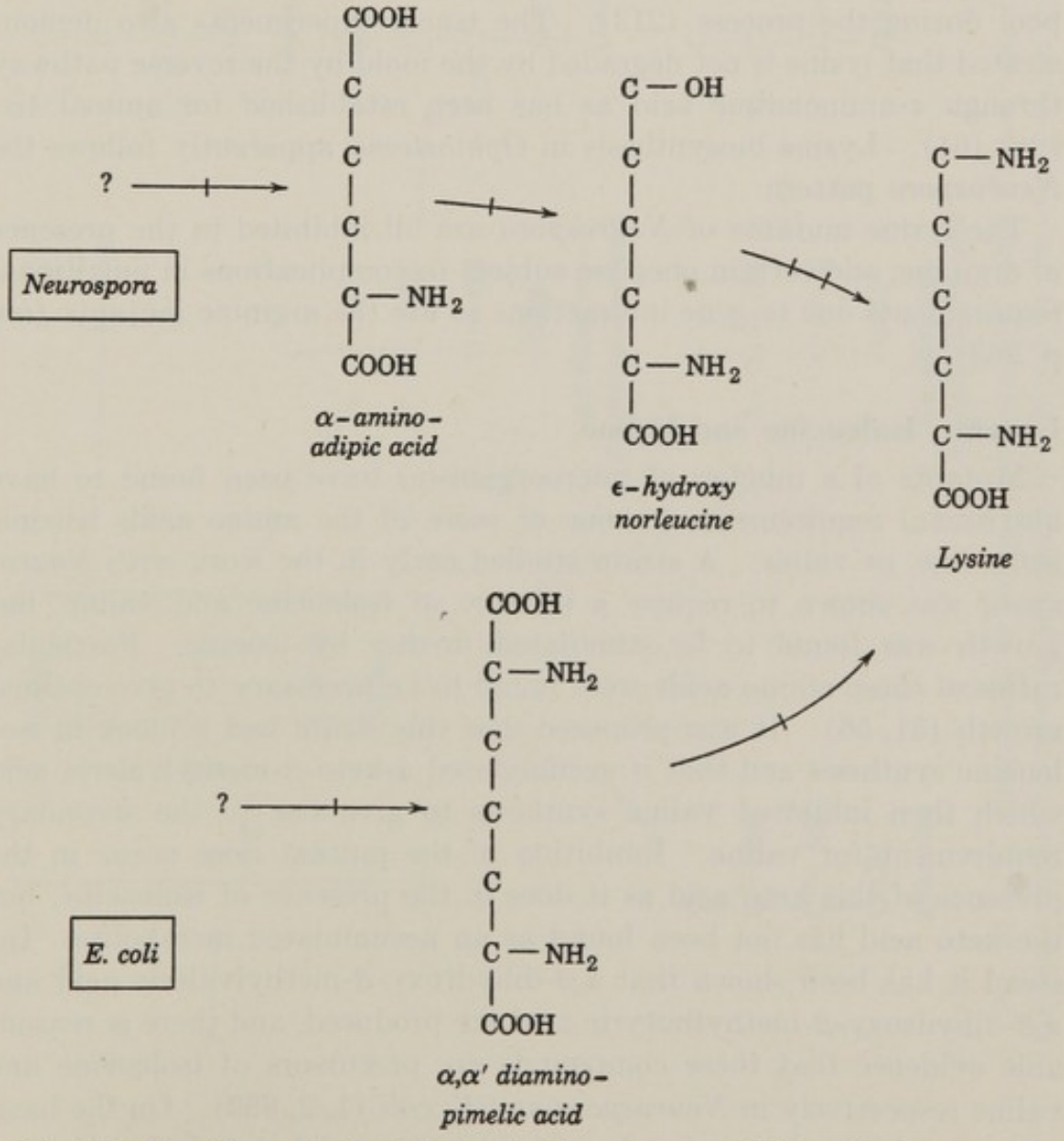


Fig. 57. The biosynthesis of lysine in *Neurospora* and *E. coli*. Two distinct pathways for biosynthesis are indicated.

seem to be the case with lysine biosynthesis in *Neurospora* and *E. coli*, both of which normally have the capacity to synthesize this amino acid starting with sugar and inorganic nitrogen. The *Neurospora* mutants that have requirements for lysine do not utilize diaminopimelic acid nor do the corresponding *coli* mutants use the designated *Neurospora* precursors. Diaminopimelic acid is accumulated by one *coli* mutant and utilized by another. In addition it has been shown that the mutant that accumulates the compound is deficient in an enzyme system that decarboxylates the substance to form lysine. On the other hand, tracer experiments with C^{14}-labeled α-aminoadipic acid have shown that this substance is indeed converted to lysine in *Neurospora* (422) although the amino nitrogen is exchanged freely with that of the general nitrogen pool during the process (213). The tracer experiments also demonstrated that lysine is not degraded by the mold by the reverse pathway through α-aminoadipic acid as has been established for animal tissues (61). Lysine biosynthesis in *Ophiostoma* apparently follows the *Neurospora* pattern.

The lysine mutants of *Neurospora* are all inhibited in the presence of arginine, and certain ones are subject to complications in nutritional requirements due to gene interactions as are the arginine mutants (see p. 263).

Leucine, Isoleucine and Valine

Mutants of a number of microorganisms have been found to have nutritional requirements for one or more of the amino acids leucine, isoleucine, or valine. A strain studied early in the work with *Neurospora* was shown to require a mixture of isoleucine and valine, but growth was found to be stimulated further by leucine. Particular ratios of these amino acids were found to be necessary to give optimal growth (51, 56). It was proposed that this strain had a block in isoleucine synthesis and that it accumulated α-keto-β-methylvaleric acid which then inhibited valine synthesis to give rise to the secondary requirement for valine. Inhibition of the mutant does occur in the presence of this keto acid as it does in the presence of isoleucine, but the keto acid has not been found as an accumulated metabolite. Instead it has been shown that α,β-dihydroxy-β-methylvaleric acid and α,β-dihydroxy-β-methylbutyric acid are produced, and there is reasonable evidence that these compounds are precursors of isoleucine and valine respectively in *Neurospora* and *E. coli* (1, 2, 659). On the basis of work with tracers it has been further proposed that isoleucine and perhaps valine have an origin in acetate and a four-carbon hydroxy-

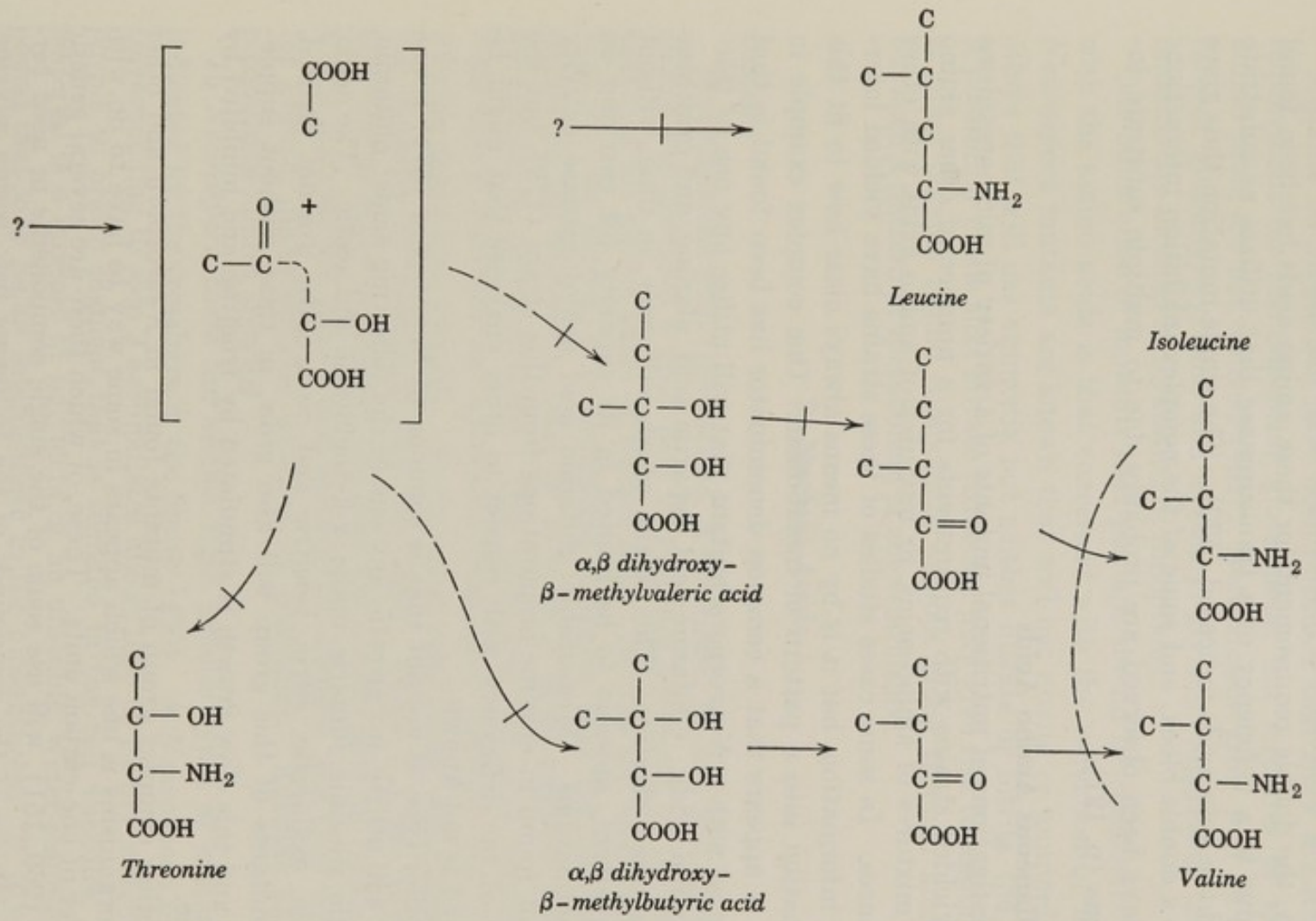


Fig. 58. The biosynthesis of leucine, isoleucine, and valine. It has been shown that a single mutation can block similar reactions in two series as indicated by the cross bar just following the keto acids that correspond to isoleucine and valine.

keto acid that also provides the carbon chain of threonine. These suggestions are summarized in Fig. 58. As indicated in the figure (by the cross bar between the reaction series giving rise to isoleucine and valine), the double requirement for these amino acids has been found to be due to a deficiency of a transaminase that utilizes as substrate both of the keto acids indicated (522). The single mutation thus gives rise to a double block, and some of the complex inhibition interactions that have been observed are probably due to multiple substrate inhibitions (p. 121).

Miscellaneous Amino Acids

Investigations of nutritional mutants of a variety of microorganisms have yielded strains with requirements for a number of other amino acids, mixtures of amino acids, or mixtures of amino acids with other substances. In some cases studies of these strains have yielded interesting information, but it is by no means always clear how to fit this information into a pattern of metabolism. One complex example in which it appears that a common denominator has been found is that concerned with a *Neurospora* mutant that will utilize any one of thirteen amino acids. Glutamic acid, aspartic acid, alanine, and ornithine are the most effective. This strain, which will grow, in time, without a supplement, appears to be limited in its capacity for carrying on transaminations. Measurable glutamic acid dehydrogenase activity was not found in enzyme preparations from this strain (178), but the extent of the deficiency with respect to other enzymes that might be involved is not known.

Another type of mutant that is common in *Neurospora* (374) is one which will utilize, for growth, any one of the following acids: glutamic, aspartic, succinic, fumaric, malic, α-ketoglutaric and acetic. The acids, pyruvic, oxalacetic, citric, isocitric and *cis*-aconitic are not utilized. The mutants of this group will also grow, in time, without supplement, and this slow growth is stimulated by fructose and inhibited by glucose.

Another complex group of mutants found in *Neurospora* and other microorganisms is one which appears in some way to have to do with transfer of one-carbon units. These, of which there are several genetic types (260, 151), will use some of the single components or need certain mixtures of the compounds of the following list: serine, glycine, formic acid, formaldehyde, histidine, methionine, adenine, and *p*-aminobenzoic acid. The common denominator here appears to be participation of these substances as donors of one-carbon fragments or par-

ticipation in the metabolism of these fragments. Information on the nature of the pattern involved is not available.

Mutants of *Glomerella* have been obtained that show a nutritional requirement for the tripeptide glutathione, but mixtures of the free amino acids are not utilized (404). Both strains grow well on a mixture of glutamyl-cysteine and cysteinyl-glycine, suggesting that the mutants are blocked in formation of dipeptides.

One other class of mutants that perhaps should be considered along with the amino acids is that concerned with the reduction of nitrate. *Neurospora* mutants are known that will use nitrite or ammonia, and others that will use ammonia but neither nitrate or nitrite (34, 298). A possibility exists that nitrate reduction may give rise to amino acids or other organic amino compounds without going through ammonia, but such a pattern of metabolism is not established.

2. Nucleic Acids

Purines

Investigations with *Ophiostoma, Neurospora, E. coli,* and *Saccharomyces* has provided a good deal of evidence pertinent to the biosynthesis of the purine bases of nucleic acid. Results are summarized in Fig. 59. As shown, it is considered probable that the biosynthesis occurs at the nucleoside level or at even a more complex level, as nucleotide or polynucleotide. This point has not been established. *Neurospora* contains an adenosine deaminase activity but not an adenine deaminase activity. On the other hand, some organisms have the capacity to deaminate the free base. Steps 1–4 are probably common to many organisms, but step 4 is known only in *Ophiostoma* where mutants are known that accumulate adenine, and they will use, for growth, diaminopurine, guanine, or guanosine. Adenine and hypoxanthine inhibit growth.

In *Neurospora* there are known eight genetic types of mutants as indicated by the crossbars in the figure (420). A *Neurospora* mutant corresponding to step 2 accumulates about 15% of its dry weight as a purple to brown pigment that is secreted in the cell vacuoles. This material is a mixture of polymers of an unstable, water-soluble compound that may be an intermediate in the biosynthesis. It is related in structure to the aminoimidazole carboxamide that is produced by *E. coli* (560) and utilized for growth by some mutants of *E. coli* (43) and *Ophiostoma* (189). Pigment accumulation in *Neurospora* was used as a label to determine the order of the mutants as already de-

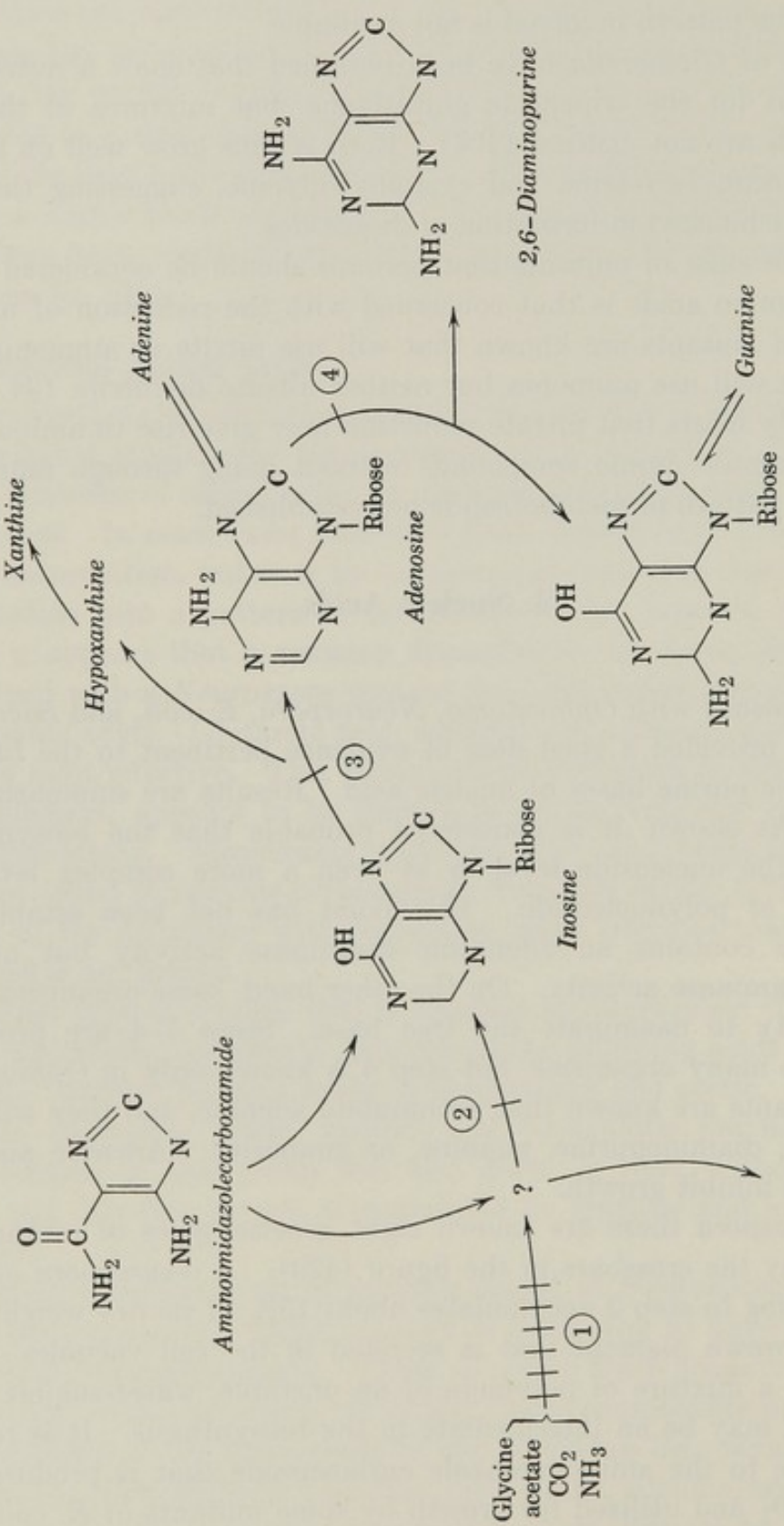


Fig. 59. Biosynthesis of the purines of nucleic acids. Genetic blocks indicated by the cross bars and circled numbers are discussed in detail in the text.

scribed for the histidine series (p. 200). As shown in the figure, all the mutant genes of over-all step 1 prevent the formation of pigment when in combination with the gene that causes accumulation.

The pigment or some of its precursors are slightly inhibitory to growth, and cultures of the purple mutant have been observed to become white but still adenine-requiring, owing to selection of a double mutant in which the second spontaneous mutation prevents the formation of the pigment. This phenomenon is like that reported for the guanine mutants of *Ophiostoma* which select for guanine-hypoxanthine double mutants as a response to inhibition by the adenine accumulated by the single guanine mutant (187). It has been pointed out that selection of secondary spontaneous mutations in such a fashion provides a reasonable basis for physiological evolution.

Pyrimidines of Nucleic Acids

As shown in Fig. 60 several genetically different pyrimidine mutants of *Neurospora* have been found (421). All the mutants will utilize uridine or cytidine with equal facility. Uracil is used adaptively by all of the mutants if the mold is grown on a medium containing NH_3, but the pyrimidine is used as efficiently and rapidly as the nucleosides

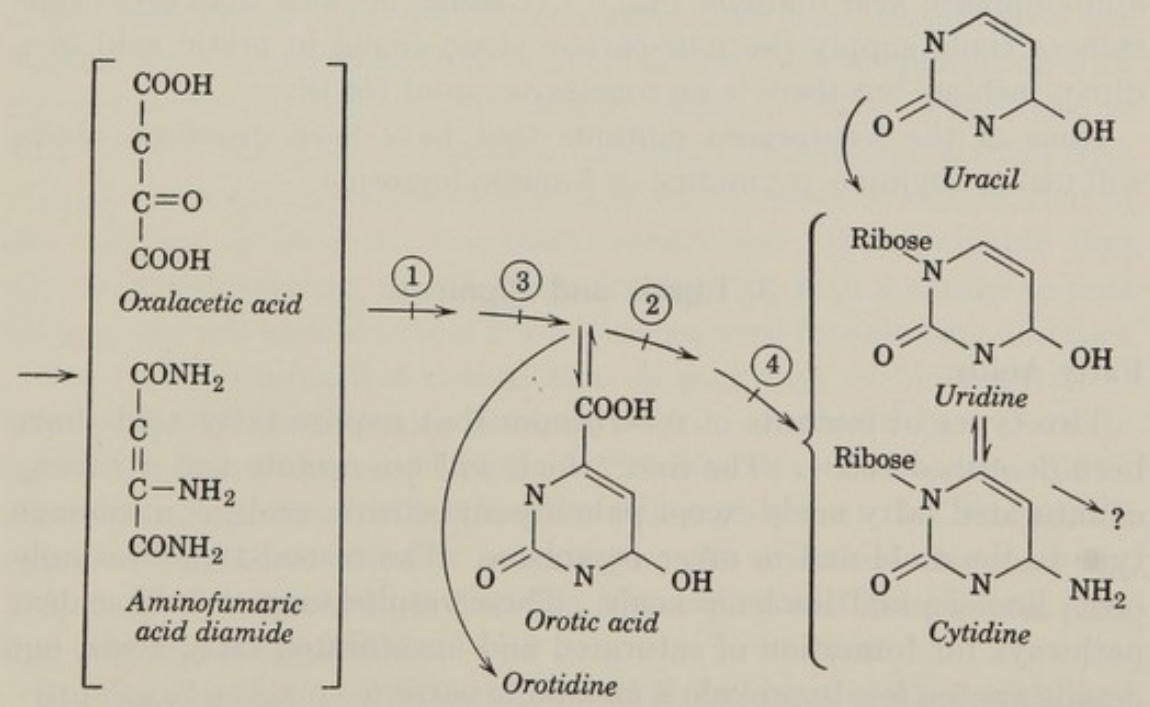


Fig. 60. Biosynthesis of some of the pyrimidines of nucleic acids. Studies of mutants have not established whether orotic acid and orotidine are true intermediates, but enzyme work has shown that orotic acid goes to orotidine phosphate and thence to the phosphates of uridine and cytidine instead of to the free nucleosides as indicated here.

on a medium containing NO_3^- as the sole nitrogen source (417). Similarly mutant 3 (Fig. 60) will use orotic acid effectively only in a nitrate medium while mutant 1 will utilize this pyrimidine in either medium. A natural riboside of orotic acid (orotidine (412)) is used only adaptively and poorly by mutants 1 and 3 and not at all by mutants 2 and 4. Both these last two strains accumulate large quantities of orotic acid and smaller quantities of orotidine during growth. It may be that neither of these compounds is an intermediate in the biosynthesis of uridine and cytidine, but they may have some other biological importance since it has been demonstrated that orotic acid is required for growth by *L. bulgaricus* (706). This nutritional requirement cannot be replaced by the pyrimidine bases or nucleosides that are known to occur in the nucleic acids. Regardless of the ultimate biochemical fate of orotic acid or orotidine it is clear that these substances are products of the biosynthesis of uridine and cytidine since double mutants of strains 1 or 3 with 2 or 4 do not accumulate the carboxylated pyrimidines (421).

The formation of orotic acid by *Neurospora* mutants is in keeping with the earlier observations that two pyrimidine mutants with partial genetic blocks are able to grow in the presence of oxalacetic acid and aminofumaric acid diamide (421). It seems obvious that these substances could supply the four-carbon chain found in orotic acid in a direct fashion, but there is no conclusive proof for this.

None of the *Neurospora* mutants that have been discussed above will utilize thymine, thymidine or 5-methylcytosine.

3. Lipids and Pigments

Fatty Acids

Two types of mutants of *Neurospora* that require fatty acids have been described (360). The first, which will use acetate and a variety of saturated fatty acids except palmitic and stearic acids, is a common type in the mold and in other organisms. The second type uses only oleic, linoleic, and linolenic acids. These results suggest independent pathways for formation of saturated and unsaturated fatty acids, but details are too few to provide a metabolic pattern.

Carotenoids

Mutants in the tomato and in *Neurospora* have provided a basis for studying the biosynthesis of carotenoids. Precise details are not yet

available, but it appears that the colorless and more hydroxylated polyenes are precursors to the more unsaturated colored polyenes (266). A number of complications of gene interaction influence biosynthesis and the processes of isomerization (p. 85).

Porphyrins

Mutants of *Chlorella* have been utilized for studies of the pathway of biosynthesis of chlorophyll (222). The following general sequence of reactions has been suggested: → protoporphyrin → Mg-protoporphyrin → Mg-vinylprotoporphyrin → Mg-vinylprotoporphyrinphytyl ester → chlorophyll *a*. Many more individual steps are to be expected, and there is good reason to believe that the biosynthesis of the iron porphyrins as in the cytochromes, other enzymes, and hemoglobin may fit into the same pattern up to the protoporphyrin stage.

4. B Vitamins

Mutants with requirements for vitamins in the B group have been found commonly in all the microorganisms that have been used for mutant selections. Metabolic patterns concerned with the formation of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and nicotinic acid are the best known, and these have been considered here in connection with the metabolism of the aromatic amino acids (p. 194). With these as with most of the other B vitamins, excepting lipothiamin, studies with mutants have yielded little information on processes of conjugation of the vitamins to form the functional coenzymes. It is probable that most of the coenzymes are too large or carry too high a charge to pass through the cell membranes of the organisms used for selecting mutants.

Very little information is available on pathways of biosynthesis of biotin, inositol, pantothenic acid, pyridoxin or riboflavin. Desthiobiotin acts as a precursor of biotin in *Penicillium* or *Neurospora* (634). *Neurospora* will also utilize biocytin (biotinyl-lysine), but the significance of this observation is not known. As discussed elsewhere (p. 173) pantothenic acid is produced in *Neurospora* and *E. coli* from pantoic acid and β-alanine.

Studies of mutants of *Neurospora* and *E. coli* have yielded significant information on the biosynthesis of thiamine (635) and lipothiamide (507). A general pattern of metabolism is presented in Fig. 61. The steps 1–3 were designated on the basis of studies with *Neurospora* mutants. The mutant corresponding to 2 was shown to accumulate

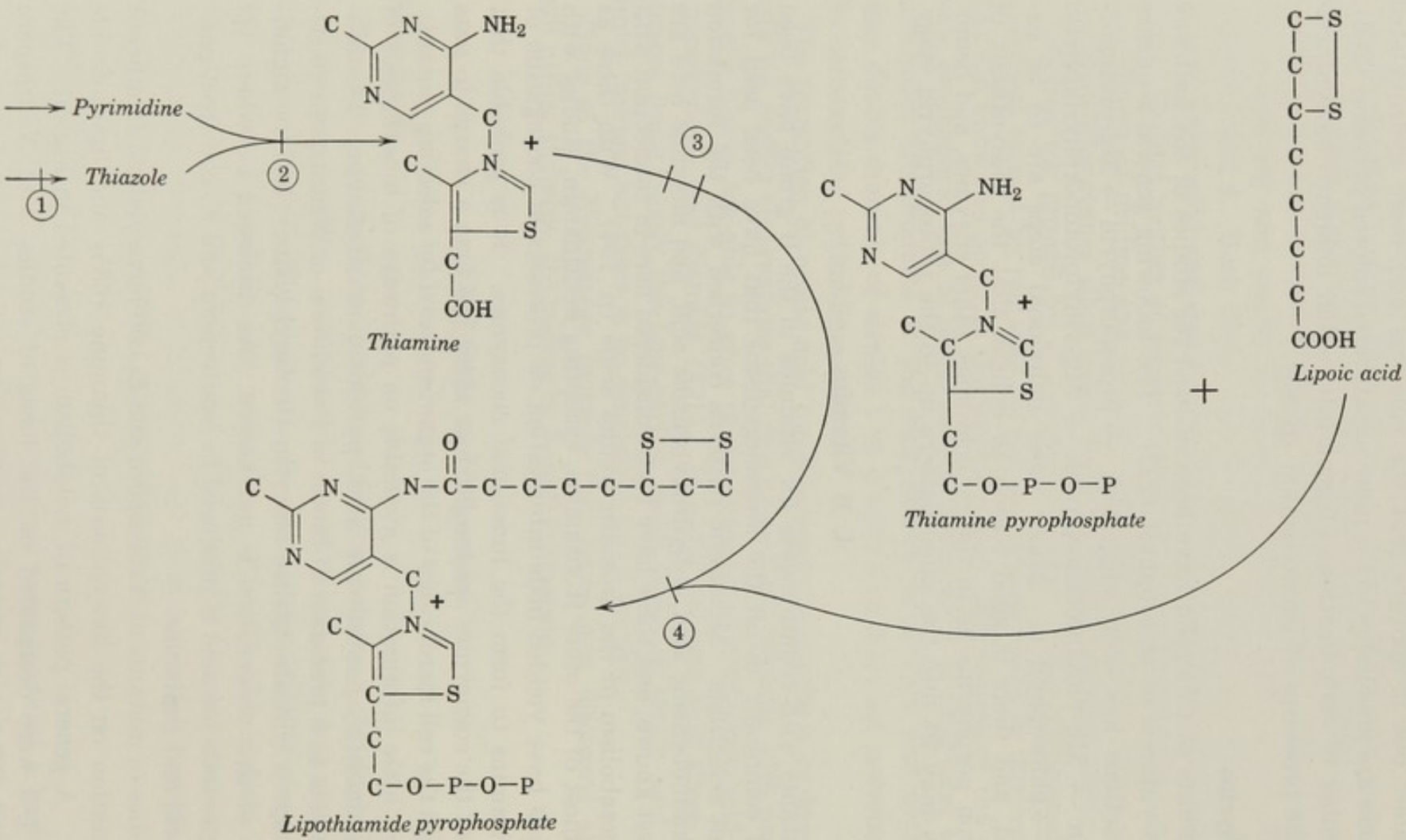


Fig. 61. The biosynthesis of thiamine and some derivatives. Apparent positions of genetic blocks in several mutants are indicated by cross bars and circled numbers.

pyrimidine and thiazole components. One of the mutants of step 3 accumulates a substance that can be utilized by 2, but this has not been isolated and it might be thiamine pyrophosphate since the *Neurospora* mutants will utilize this compound in the place of thiamine. Mutants 3 have an abnormally high requirement for thiamine and will use thiazole plus pyrimidine. It was therefore suggested that they are mutants with partial blocks with a requirement for a thiamine con-

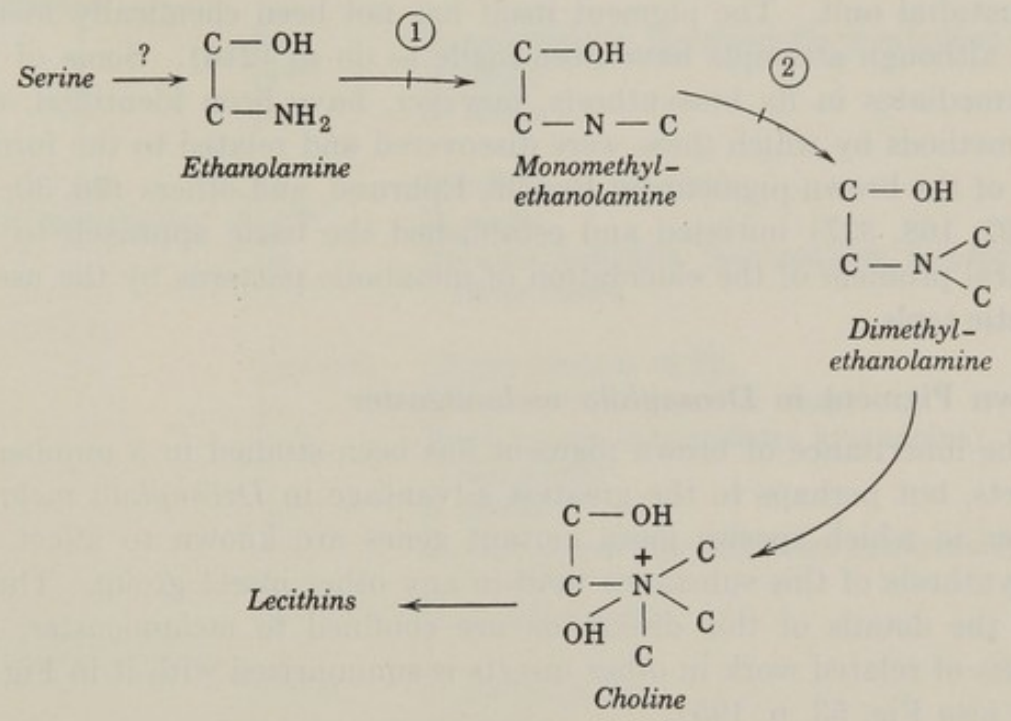


Fig. 62. The biosynthesis of choline.

jugate. That this is lipothiamide pyrophosphate as shown in Fig. 61 has not been established for *Neurospora.*

Several steps in the biosynthesis of choline in *Neurospora* have been established as shown in Fig. 62 (297). Monomethylethanolamine was shown, by isolation, to be accumulated by a mutant at step 2. This mutant will utilize the compound it accumulates as well as the dimethyl derivative and choline. The accumulation is therefore a result only of a change in the relative reaction rates of steps 1 and 2. These choline mutants will both utilize acetylcholine, arsenocholine, phosphoryl choline, dimethylethyl hydroxyethyl ammonium chloride, triethylcholine, and methionine. The utilization of methionine can be assumed to be due to a sparing action for methyl groups if it is considered that the choline mutants have a partial capacity for choline synthesis. Lecithins have been isolated from *Neurospora,* but other conjugates

are not known. It is worthy of note that the *Neurospora* mutants provide a basis for one of the best of known assays for choline.

5. Insect Eye Pigment

The brown pigment found in the ommatidia of the compound eyes of many, if not all, insects is a complex consisting of protein and pigment proper deposited in granular form in various parts of the ommatidial unit. The pigment itself has not been chemically identified, although attempts have been made to do so (216). Some of the intermediates in its biosynthesis, however, have been identified, and the methods by which these were discovered and related to the formation of the brown pigment by Beadle, Ephrussi, and others (26, 30, 31, 41, 92, 168, 327) initiated and established the basic approach to the general problem of the elucidation of metabolic patterns by the use of genetic tools.

Brown Pigment in *Drosophila melanogaster*

The inheritance of brown pigment has been studied in a number of insects, but perhaps to the greatest advantage in *Drosophila melanogaster* in which species more mutant genes are known to affect the biosynthesis of this substance than in any other insect group. Therefore the details of this discussion are confined to *melanogaster,* but results of related work in other insects is summarized with it in Fig. 63 (see also Fig. 53, p. 195).

The compound eye of *Drosophila* sp. actually contains two types of pigment, the brown and the red. The red is not directly related to the brown, and it occurs, as far as known, only in the family Drosophilidae. The mutant genes to be considered in the following have little or no effect on the red pigment component of the eyes, and therefore no further mention will be made of it here, although it is considered in conjunction with the brown pigment in Chapter 10.

Four recessive, non-allelic genes are known to effectually block the formation of brown pigment in *D. melanogaster: vermilion* (*v*), *cinnabar* (*cn*), *scarlet* (*st*), and *cardinal* (*cd*). The phenotypes of flies homozygous for any one of these genes are almost identical. But that the phenotypes result from different causes has been established by a variety of different methods: (1) by transplantation of parts between different genotypes, (2) by feeding pigment precursors to the larvae, and (3) by direct chemical analysis. The successful application of these methods to higher animal forms such as insects illustrates that

the analysis of metabolic pathways with mutant organisms need not be restricted to microorganisms. The transplantation experiments involve the removal of those parts of the larvae which are to go directly into the formation of the adult during pupation—the so-called imaginal discs or adult tissue anlage, and their implantation into the haemocoels

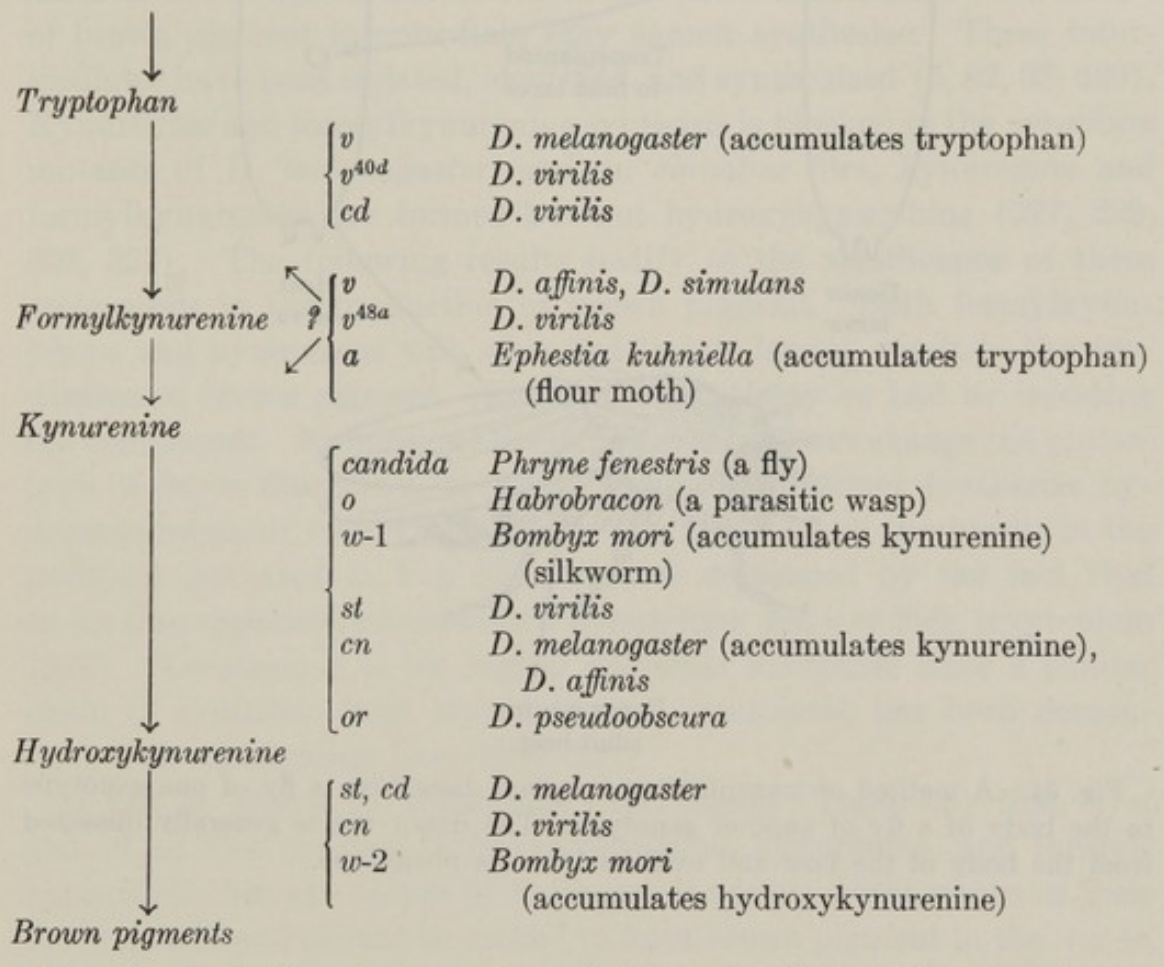


Fig. 63. Production of brown pigment in insects. Each of the steps in the series is interrupted by gene mutations in a number of different insects. (Data from 31, 34, 92, 228, 326, 327, 365, 495, 629, 663.)

of other larvae which act as hosts (Fig. 64). The host larval and pupal environment permits the donor tissue to develop to approximately the adult state. Thus, if that part of a larval imaginal disc known as the eye anlage is transferred to a host larva during pupation, the eye anlage will differentiate and form an eye containing pigment, in the abdomen of the developing host adult. The eye, however, does not evert and present a completely normal appearance. It must therefore be dissected to determine the amount of pigment produced. Table 28 gives the results of transplants between donors and hosts of indicated

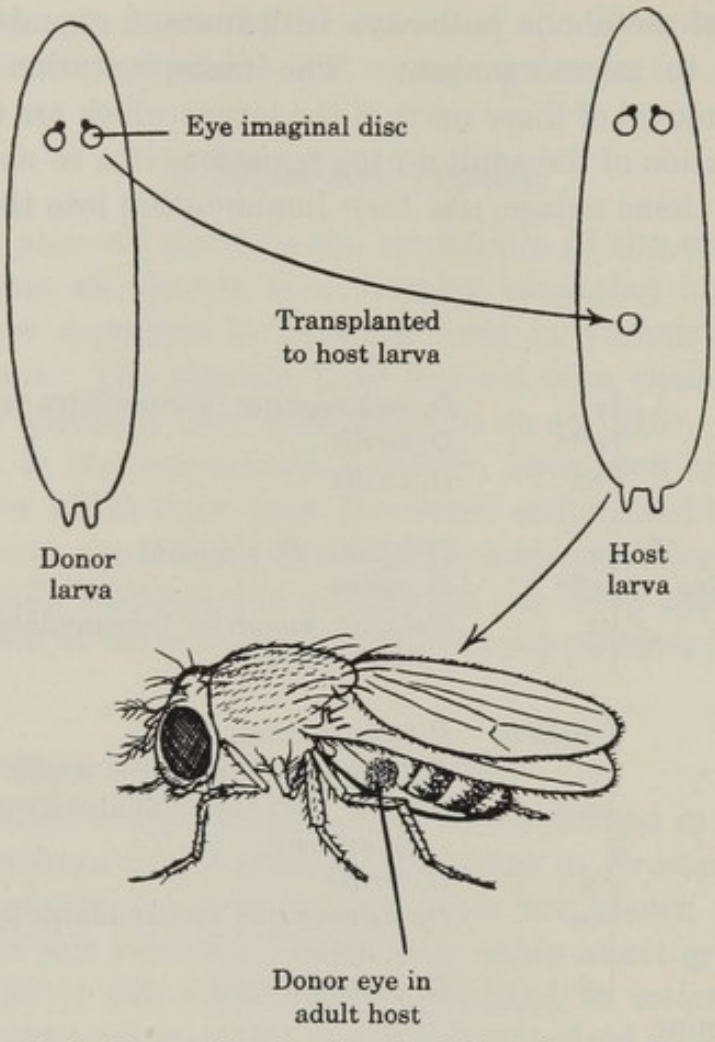


Fig. 64. A method of transplanting imaginal discs from a fly of one genotype to the body of a fly of another genotype. The donor eye is generally dissected from the body of the host and examined for its phenotype.

Table 28. Pigment Formation in *Drosophila* Eye Transplants

Donor Genotype	Host Genotype	Formation of Pigment in Donor Eye Transplant
+/+	+/+	+
v/*v*	+/+	+
cn/*cn*	+/+	+
+/+	*v*/*v* or *cn*/*cn*	+
v/*v*	*cn*/*cn*	+
cn/*cn*	*v*/*v*	−
cn/*cn*	*cn*/*cn*	−
v/*v*	*v*/*v*	−
cn/*cn* or *v*/*v*	*st*/*st* or *cd*/*cd*	+
st/*st* or *cd*/*cd*	+/+ or *v*/*v* or *cn*/*cn*	−

genotype in terms of the formation of brown pigment. It will be noted that *v/v* and *cn/cn* eye anlage develop brown pigment in +/+, *st/st*, and *cd/cd* host, indicating that a diffusible intermediate not formed by the mutant anlage is supplied by the hosts. The fact that *v/v* anlage develop pigment normally in *cn/cn* hosts, but that *cn/cn* anlage in *v/v* hosts do not do so, further indicates that the mutants differ in the kind of brown pigment intermediate they cannot synthesize. These intermediates have been isolated, identified, and synthesized (5, 82, 83, 639). Kynurenine and formylkynurenine synthesis is blocked in the *vermilion* mutants of *D. melanogaster,* and, in *cinnabar* flies, kynurenine and formylkynurenine are formed but not hydroxykynurenine (227, 228, 326, 327). The following results testify to the significance of these compounds in the production of brown pigment. Both formylkynurenine and kynurenine will, when fed to *v/v* larvae, result in the production of brown pigment. Identical results may be had by injecting the compounds. Kynurenine itself, however, will not change the phenotype of *cn/cn* flies which therefore presumably cannot synthesize hydroxykynurenine from kynurenine. The existence of the blocks in the positions indicated in Fig. 63 is further evidenced by the fact that *cn/cn* flies apparently accumulate kynurenine and *v/v* flies, tryptophan (227). Tryptophan is the logical precursor substance since a similar chain of synthesis from tryptophan to kynurenine has been demonstrated in mammalian liver (330, 331).

It can be shown by transplant experiments (Table 28) that *st/st* and *cd/cd* flies produce hydroxykynurenine, kynurenine, and formylkynurenine, but eye anlage of these genotypes are autonomous in their development and cannot be caused to form brown pigment in the +/+, *v/v* or *cn/cn* hosts. The intermediate compounds required by these mutants are possibly not diffusible. There is at present no clue as to what these intermediates may be.

Homologies with Other Insect Species

That metabolic patterns are similar in diverse groups of organisms has been well established by biochemists, and that mutations in different organisms can cause similar disturbances in these patterns has also been well established. The homologies of these mutant genes are, however, difficult if not impossible to establish, except in those taxonomic groups in which a number of species have been extensively investigated genetically. One of the few such groups is the insect genus *Drosophila.* As Sturtevant and Novitski (629) have shown, an analysis

of the known mutants of a number of the species of this group reveals that chromosomes can be homologized. Thus the X chromosomes of most species that have been investigated show similar gene mutations in so far as these mutant genes affect the phenotype. This homology has been brought to the biochemical level by the demonstration that at least five species (Table 29) have a mutant gene on the X chromo-

Table 29. Genes with Homologous Effects on the Phenotypes of Five Different Species of *Drosophila*. Chromosomal Associations as Indicated on the Basis of Homologies with *Drosophila Melanogaster*

	X chromosome	2R
melanogaster	*v*	*cn*
simulans	*v*	—
pseudoobscura	*v*	*or*
affinis	*v*	*cn*
virilis	*v*	*st*

some designated as *vermilion,* which does not form kynurenine. Four of these same five species (Table 29) have a mutant gene located on a chromosome element homologous to the right arm of the 2nd chromosome of *D. melanogaster* which causes the suppression of formation of hydroxykynurenin as does *cinnabar* in *melanogaster.* In Fig. 63 a number of other insect species, some of them in other orders than *Drosophila,* are indicated as having genetic blocks similar to the *vermilion* or *cinnabar* of *melanogaster* and its relatives. Chromosome homologies cannot, however, be established between these other species because of the considerable degree of unrelatedness. It is of interest, though, that in all the insects so far investigated, in which mutant genes affecting brown eye pigment have been found, similar disturbances of a similar metabolic pattern have been related to the mutant genes.

6. Genetic Blocks

The patterns of metabolism as they have been presented in this chapter are very much oversimplified at the level of the individual reactions involved. They have been written, for convenience, with different steps indicated as in the reaction A $\rightarrow$ B shown in Fig. 65*a*, where the cross bar and "Gene 1" designate the apparent position of the block so far as a mutant phenotype is concerned. It is necessary, however, to inquire into the meaning of the term "genetic block," on a more dynamic biochemical basis, and, taking into account the facts and principles discussed in Chapters 5 and 6, it is apparent that the

system shown in Fig. 65*b* provides a more nearly correct description of the *in vivo* situation than that of Fig. 65*a*. Here, with the genetic block expressed phenotypically, by choice, as a reduced rate of appearance of available B, it is shown that this reduced rate can come about by a change in the rate of any one of a large number of other

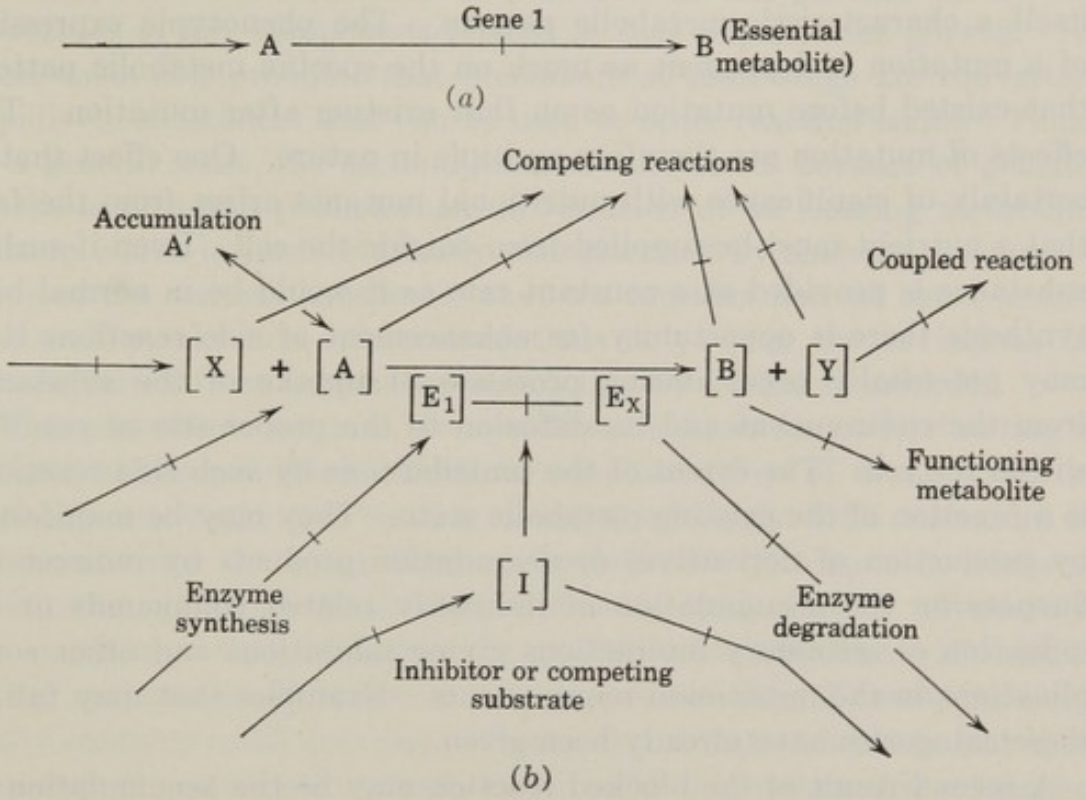


Fig. 65. A diagrammatic representation of a genetic block. (*a*) Simplified designation. (*b*) Generalized pattern. The cross bars indicate some possible positions of change in reaction rates that can give rise to a change in the rate of reaction A → B.

reactions, as indicated by the cross bars on the various arrows in the figure.

A reaction pattern can be somewhat simpler or a good deal more complex than that shown here as a generalized case as already discussed in this and earlier chapters. But the reaction rate in question is dependent on approximately steady state concentrations of the reactants and products designated in Fig. 65*b* as $[X]$, $[A]$, $[E_1]$, $[E_x]$, $[I]$, $[B]$, and $[Y]$. These concentrations are in turn dependent on relative rates of formation of components and diversion into side reactions. $[E_1]$ and $[E_x]$ are included to indicate the possibility of existence of metabolically different forms of an enzyme, and the coupled reaction is shown to emphasize the necessity for considering

energetics. All these points have been discussed in connection with isolated enzyme systems, and it is considered that the principles so derived are qualitatively applicable to *in vivo* reactions even though many of these no doubt occur through sequences of organized steps with activated intermediates remaining attached to enzymes.

The point of principal emphasis here is that a genetic block is in itself a characteristic metabolic pattern. The phenotypic expression of a mutation is dependent as much on the specific metabolic pattern that existed before mutation as on that existing after mutation. The effects of mutation are therefore multiple in nature. One effect that is certainly of significance with nutritional mutants arises from the fact that a nutrient must be supplied from outside the cell. Even if such a substance is provided at a constant rate as it would be in normal biosynthesis there is opportunity for enhancement of side reactions that may potentially occur during processes of uptake of the substance from the environment and its diffusion to the proper site of reaction within the cell. The extent of the contributions by such side reactions is a function of the existing metabolic state. They may be manifested by production of derivatives or degradation products by indirect influences on the accumulation of indirectly related compounds or by induction of secondary interactions giving inhibitions and other complications in the nutritional requirements. Examples that may fall in these categories have already been given.

A second result of the blocked reaction may be the accumulation of some substance fairly closely related chemically to the product initially observed to be affected. Numerous examples of accumulations of this kind have been described in this chapter. It is particularly important to recognize that the production of such a substance in quantity is a result of a shift in material balance, and it is neither a new event nor is the substance formed usually an inert by-product.

As one example (Fig. 53), it has been shown that a *Neurospora* mutant (10575) which will utilize indol or tryptophan for growth accumulates anthranilic acid to a concentration of about 0.1 mg/ml in the culture fluid under a given set of conditions. In the presence of an adequate supply of sugar this concentration is maintained over a period of several days, but as soon as the carbon source is exhausted the anthranilic acid disappears in a few hours in the presence of oxygen. Added anthranilic acid is also oxidized quite rapidly by a mold pad that is no longer producing it. The oxidative system may be adaptive, but the maintenance of a constant concentration of the compound can

also be accounted for by assuming a low affinity for the substance by the oxidative enzyme. This accumulated substance is therefore turned over at a fairly rapid rate, and the concentration of the compound is limited by the effectiveness of the degradation process. Some substances that are produced as the result of genetic block do appear to be inert, but more often than not mutants do not produce detectable quantities of any compounds related to that required for growth. In these cases it is probable that precursors at some stage are converted rapidly to substances that can be used in other reaction series. Thus, in a general sense, the accumulation of substances because of genetic blocks is merely a phenomenon of variation of an existing metabolic pattern which in itself is already characterized by showing accumulations of the various substances that we consider normal cell constituents. We take note of accumulations only when the deviations in concentration from normal are observable by available methods.

This general picture provides a reasonable basis for understanding a variety of other multiple effects of mutation. One of these, which is probably quite common but has received relatively little attention, is concerned with the accumulation of substances that have no obvious relation to the biosynthetic series that seems most directly affected by the mutation. Two examples are available from studies with nutritional mutants of *Neurospora*. It was shown that *Neurospora* normally contains small amounts of hexapoly- or metaphosphate and that a variety of nutritional mutants of the mold accumulate this substance (303). Some data are summarized in Table 30. The accumulations of

Table 30.* The Accumulation of Acid Labile Polyphosphate by *Neurospora* Mutants †

Values are given in milligrams per gram wet weight of mold.

Mutant Number	Nutrient Supplied	Acid Labile Phosphate, mg per gm
4545	Lysine	5.5
37301	Pyrimidine	6.8
55701	Lethal temperature mutant	4.9
65001	Indol	15.2
65001	Tryptophan	15.0
65001	Kynurenine	13.2
65001	Hydroxyanthranilic acid	3.4
65001	Nicotinamide	2.6

* From Houlahan and Mitchell (303).

† Sixteen other strains including wild types and various mutants yielded polyphosphate in the range 0.9 to 1.7 mg per gm.

this substance are clearly associated with specific gene mutations, but it is not at all clear how or why the compound is produced. It should be noted that the polyphosphate is formed in great excess by the tryptophan mutant (65001) only when it is grown in the presence of two (tryptophan and kynurenine) of the four substances listed that can be utilized for growth. Perhaps, in this case, the accumulation is a by-product of the functioning of the tryptophan cycle (Fig. 53, (262)) since, of the four compounds tested, only tryptophan and kynurenine participate in the cycle.

A picture somewhat similar to that of polyphosphate accumulation has been obtained from studies of the formation of pyruvate and α-ketoisovalerate by a number of *Neurospora* mutants (417). Data are summarized in Table 31. Here again there is no good reason to

Table 31.* Dinitrophenylhydrazones of Pyruvic Acid and α-Ketoisovaleric Acid Obtained from a Variety of *Neurospora* Mutants †

Values are given in grams per 15 liters of culture medium.

Mutant Number	Substance Required	Total Hydrazones, gm	Purified Hydrazones	
			Pyruvate	Ketoisovalerate
4545	Lysine	6.5	0.4	5.2
37101	Lysine	11.0	5.0	5.0
C84	Histidine	4.5	3.0	1.1
C94	Histidine	12.0	5.0	6.0
29997	Arginine	6.0	0	4.5
263	Pyrimidine	5.5	4.5	0
37301	Pyrimidine	4.5	4.3	0
36601	Pyrimidine	3.0	2.6	0

* From Mitchell (417).

† Wild-type strains and 15 mutants with various nutritional requirements have been found not to accumulate these keto acids in significant quantities under the growth conditions used.

believe that there is any very direct connection between the nature of the substance accumulated and the structures of the nutrients required by the various mutants investigated. Since the production of these keto acids is a function of the mutant genes present and not a function only of the presence of the compound required for growth, these accumulations do represent multiple effects of gene mutations.

In both these examples good evidence has been obtained to demonstrate that the mutants of each group that show the accumulations do not carry a common mutation that gives rise to the phenomena. It must be concluded that each of a group of mutants with different

nutritional requirements can so affect the pattern of metabolism as to give rise to a common accumulant. There is not necessarily a common mechanism for this since there are many conceivable ways in which the concentration of a metabolite can be affected.

Very probably related to the processes of altering composition through changes of relative reaction rates by mutation is the phenomenon of complication of mutational effects by environmental factors that had little or no observed influence on the pre-mutation pattern of metabolism. Some good examples are given in this chapter in the sections on metabolic patterns related to the nutritional mutants with requirements for arginine, histidine, lysine, isoleucine-valine, aromatic compounds, riboflavin, and others. In these cases growth is inhibited markedly by substances or mixtures of substances (normal metabolites) that did not significantly affect the growth of the strains from which the mutants were derived. It is not known whether these inhibitions arise because of the necessity of supplying the compounds needed for growth from the external environment or from some other effect of the various mutations on the pattern of metabolism, but these results represent clear examples of multiple effects of mutation.

An even more striking demonstration of the significance of inhibitions in relation to genetic blocks is provided by some other investigations with *Neurospora* (146, 147). Mutants of the fungus were selected deliberately on the basis of inhibition by normal metabolites rather than on the basis of requirements for normal metabolites. Several such strains were obtained, and two have been studied extensively. Both of these mutants are phenotypically wild type when grown in minimal medium, but in the presence of threonine one of the strains grows to only a very limited extent at 35°C whereas the other shows only slight growth in the presence of histidine. The histidine inhibition is relieved by the addition of any one of several amino acids. On the whole this mutant, in the presence of histidine, has a phenotype very similar to the nutritional mutant described earlier in this chapter (p. 206). The mutant showing threonine inhibition has been studied in greater detail and the following facts ascertained:

1. At 35°C, in the presence of threonine, the mutant requires for growth methionine, homocysteine, homoserine, or sulfanilamide. Choline plus the thiazole fragment of thiamine stimulate. Choline in high concentrations is inhibitory in the absence of threonine, and this inhibition is relieved by thiamine.

2. At 30°C the mutant is not inhibited by threonine or choline and it has a wild phenotype at this temperature.

3. At 25°C the mutant grows about half as well as wild type and it is stimulated by methione or thiazole plus choline. At this temperature it is inhibited to only a small extent by threonine.

4. At 18–20°C the mutant is not inhibited by threonine or choline, but its growth rate is limited, and it is greatly stimulated by thiazole.

These characteristics of this mutant of *Neurospora* describe a striking shift in a metabolic pattern due to changes in the environmental temperature. The precise changes that occur are not known, but it is clear that the nutritional requirement of a mutant is dependent simultaneously on gene action and environment. The functioning of a particular gene in producing a particular genetic block can be quite indirect. Many other examples similar to these are discussed in the chapters on environment and on gene interactions.

7. Some General Conclusions

A living cell is a highly organized unit characterized at the biochemical level by a capacity for carrying out a remarkable number of chemical reactions continuously and in such a fashion that the composition of the cell itself is dependent on the relative rates of these reactions. Genes exert a controlling influence on reaction rates, and their individual effects extend as far as reaction interdependence. The observed effect of a gene mutation is dependent on the choice of criteria and the tools available to the observer. In this chapter there have been presented a number of reaction series or portions thereof which are considered to proceed in certain living cells. These results were derived for the most part from studies of mutants that were selected on the basis of whether or not they had nutritional requirements, so this is their most obvious characteristic. Only a few of them have been examined to an appreciable extent for accompanying metabolic changes expressed in other ways. Nevertheless, this application of mutants and genetic methods in studies of metabolic pathways is an extremely useful one which may eventually provide enough information to become the backbone of a unified picture of the metabolic interrelationships of a large number of cellular components. It is this broad picture showing all of the biochemical reactions including all reactants and products that must be the ultimate goal of this type of work since herein lies the basis for a biochemical understanding of gene interaction, cytoplasmic inheritance, development, and perhaps gene function itself.

CHAPTER 9

Allelism, Allelic Interaction, and Pseudoallelism

Allelic genes were defined in Chapter 1 as those which produce phenotypic differences but can be shown by breeding tests to occupy equivalent loci on homologous chromosomes. This is a definition based on experimental evidence and refers back in its origin to the concept of the Mendelian allelomorphic factors or contrasting characters which segregate at the time of reproduction. The only assumption implicit in it is that the gene is a unit not reduced by crossing over.

Proof of allelism between genes is obtainable only by means of breeding tests with sexually reproducing organisms. If the organism is a diploid, these tests require first that strains be produced by inbreeding which are homozygous for the genes suspected of allelism. The homozygotes of different genetic constitution are then crossed and the F_1 hybrids inbred. The second generation (F_2) is examined for the phenotypic ratios which should not deviate significantly from a 1:2:1 ratio (for incomplete dominance) or a 3:1 ratio (for complete dominance of one of the genes), if the genes are allelic. A significant deviation from these ratios shows that the genes are non-allelic, and that the character is due to two or more non-allelic genes, or that some type of chromosomal aberration is involved. In haploid organisms such as *Neurospora* the expected ratio from a cross between strains carrying different alleles is 1:1.

Many gene loci are known by only two alleles, one being the "wild type," standard, usually a dominant, and the other a recessive "mutant" gene. However, there frequently occur *multiple allelic series*, which are series of seemingly allelic genes producing anywhere from 3 on up to 20 or even 80 distinguishable phenotypes (as for example the genes determining some of the cellular antigens in cattle discussed on p. 94).

Multiple alleles can be tested only two at a time in test crosses. If three or more of the "alleles" are found in the same individual, it means that some are non-allelic.

1. Phenotypic Effects of Allelic Substitutions

One of the earliest attempts to explain genetic differences in phenotype was that of Bateson and Punnet (see Bateson (19)) who postulated the "presence-absence theory" stating that the dominant phenotype was due to the presence of the gene whereas the recessive phenotype results from its absence. This theory became inadequate when multiple allelic series and the phenomenon of dominant mutant phenotype caused by a heterozygous deficiency were discovered. However, it still persists in a modified form to explain situations in which a recessive allele when homozygous produces none of the type effect produced by the dominant. For example, in the inheritance of pigmentation, the albino, or colorless animal or plant, may be assumed to be due to an absence of a gene determining pigmentation, or the presence of an allele which is inactive in forming pigment. It is possible to show that either of these cases may hold.

The recessive mutant characters: *acheate, yellow* (444), *roughest*^{2} (*rst*^{2}) (167), and *white* (469) in *Drosophila melanogaster* may be produced either by homozygous visible deficiencies, or by gene mutations. The deficiencies can be shown by cytological analysis of the salivary gland chromosomes to include the loci of the + genes which mutate to the mutant condition. Thus a deficiency in the X chromosome at the white eye locus, or a point mutation ($w^+ \rightarrow w$) will in either case result in recessive white eyes showing no trace of reddish eye pigment. The gene mutation can be differentiated from what otherwise might be a minute, invisible deficiency by showing that reverse mutations to an "active" allele are possible as described in Chapter 3 (p. 69).

Maize is another organism besides *Drosophila* in which an undoubted deficiency has been shown to produce a mutant phenotype (389, 597) (see Fig. 66). Similar aberrations probably occur in other organisms as well.

Since similar phenotypic changes may be attained by either means, it is necessary only to modify the original presence-absence theory by adding the possibility of the gene's becoming inactive. Genes which appear to be completely inactive with respect to the production of type effect are frequently termed *amorphs* (443). The term will be used

here in its widest sense; i.e., an amorph may be either an allele determining absence of type effect, or the absence of a gene, for it is quite impossible to distinguish between the two conditions in most organisms. It is probable, however, that most amorphs are not deficiencies, for deficiencies are usually lethal homozygous; the ones described above are rare exceptions, and, therefore, inactivity is probably more frequent than absence.

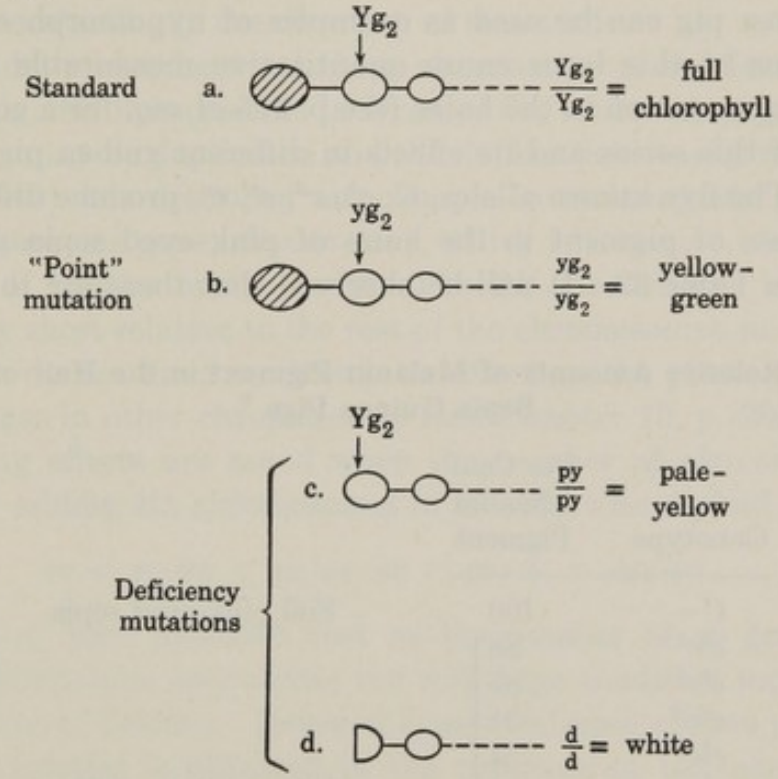


Fig. 66. A point mutation and deficiencies in maize which produce recessive mutant phenotypes. After McClintock (389). The phenotype *yellow-green* is produced by a point mutation. The phenotypes *pale-yellow* and *white* are produced by deficiencies at the tip of the chromosome. Only *white* involves the loss of the Yg_2 locus.

It must be emphasized that the term "inactivity" as applied to a gene is a description of a phenotypic effect, and, although it implies that the gene is inactive *per se*, there is no way of proving this except when deficiencies are involved. The apparent inactivation may involve only one aspect of the gene's function, and it may theoretically still be active in producing other effects in the organism. The allelic genes a^k and a both produce a recessive, colorless larval skin in the moth *Ephestia kuhniella*, but a^k/a^k adults have brown eyes and a/a adults, red eyes. The wild type a^+/a^+ larvae are pigmented and the adult eyes are black (92). Therefore, a^k and a are both amorphs with

respect to larval pigmentation but not with respect to pigmentation of the adult eye.

In contrast to the amorph, the gene without apparent activity toward producing type effect, there exist recessive genes producing type effect characteristic of the standard, + allele, but less of it. These, termed *hypomorphs* by Muller (443), appear to produce a quantitatively different phenotype from the + allele. Certain alleles in the *albino* series of the guinea pig can be used as examples of hypomorph effect, since substitutions at this locus cause quantitative measurable changes in melanin pigmentation in the hairs (see p. 275 *et seq.* for a complete description of this series and its effects in different guinea pig coat color strains). The five known alleles, C, c^k, c^d, c^r, c^a, produce different relative amounts of pigment in the hairs of pink-eyed sepia guinea pigs as shown in Table 32. It will be observed that there are three general

Table 32. Relative Amounts of Melanin Pigment in the Hair of Pink-Eyed Sepia Guinea Pigs *

Genotype	Per Cent Melanin Pigment	
$C-$	100	Full pink-eyed sepia
c^kc^k	88	
c^kc^d	65	
c^dc^d	31	
c^kc^r	54	
c^kc^a	36	Intermediate sepia
c^dc^r	19	
c^dc^a	14	
c^rc^r	12	
c^rc^a	3	
c^ac^a	0	No melanin

* From Wright (712).

levels: zero pigmentation in c^ac^a animals, full pigmentation in the presence of dominant C, homozygous or heterozygous, and intermediate levels in the presence of c^k, c^d, c^r, homozygous, and in various heterozygous combinations with one another. The allele, c^a, is an amorph, and the intermediate ones are clearly hypomorphs, causing the production of less pigment than the dominant standard, C.

One explanation of hypomorph and amorph activity in the guinea pig might be that they are acting against the standard (intense pigmentation) by inhibiting the production of type effect—the inhibition

of the hypomorphs being incomplete, that of the amorph complete when homozygous. This interpretation requires that the *C* series of genes are not involved in the production of melanin in a positive way, but negatively by controlling the production of an inhibitor. Thus the complete dominant, *C*, would be an inactive allele and the amorph, c^a, the allele with most activity. The data in Table 32 are not inconsistent with this interpretation. An alternative explanation would be that the hypomorphs are acting in the direction of standard.

A test to determine between these alternatives is not feasible with the guinea pig, but it is possible in *Drosophila*, for in this organism the chromosomes may be duplicated and the duplications easily recognized.

Schultz (549) has described an example in *melanogaster* involving the 4th chromosome gene, *shaven* (*sv*), which is recessive, and reduces the number of bristles on the body. The 4th chromosome in *melanogaster* is very short relative to the rest of the chromosomes, and changes in its dosage do not have so drastic an effect on the phenotype as do dosage changes in other chromosomes (see Chapter 10, p. 280 *et seq.*). The following effects are noted when the number of *shaven* genes is increased by adding 4th chromosomes in an otherwise diploid fly:

$$sv < sv/sv < sv/sv/sv < sv/+ = +/+$$

The symbol $<$ here indicates that as the number of *sv* genes is increased the phenotype approaches the wild-type condition more closely by the addition of bristles. Hence the most extreme *shaven* phenotype with fewest bristles is obtained in the presence of but one *sv* gene. From this it is difficult to avoid the conclusion that the *shaven* gene is acting in the direction of the standard allele, sv^+, but to a lesser degree. As might be expected, it is possible to add too many *sv* genes and obtain a fly with more bristles than wild type. Thus flies of the genotype *sv/sv/sv/sv* have extra bristles.

Similar positive activity toward type effect by mutant alleles is demonstrable in several other cases in *Drosophila* such as the alleles at the bobbed locus (603, 632) and the white locus (443). In both of these, duplication of the whole chromosome or a portion bearing the mutant allele makes the phenotype more normal, and, as would be expected, the mutant allele present once (hemizygous due to a deficiency of the part including the gene, or all of the homologous chromosome) gives the most extreme mutant effect.

An allele can be assigned to the hypomorph category with certainty only if it fulfills the conditions stated above as determined by duplication and deficiency tests. These tests are practicable only in a very

few organisms. However, since many of the genes in *Drosophila*, maize, and other plants give an increasing effect toward type with increase in dosage, it may be assumed that the occurrence of hypomorphic recessives is relatively common in all organisms.

Occasionally it is found that certain genes produce a type effect when homozygous or in combination with an allelic amorph, but behave

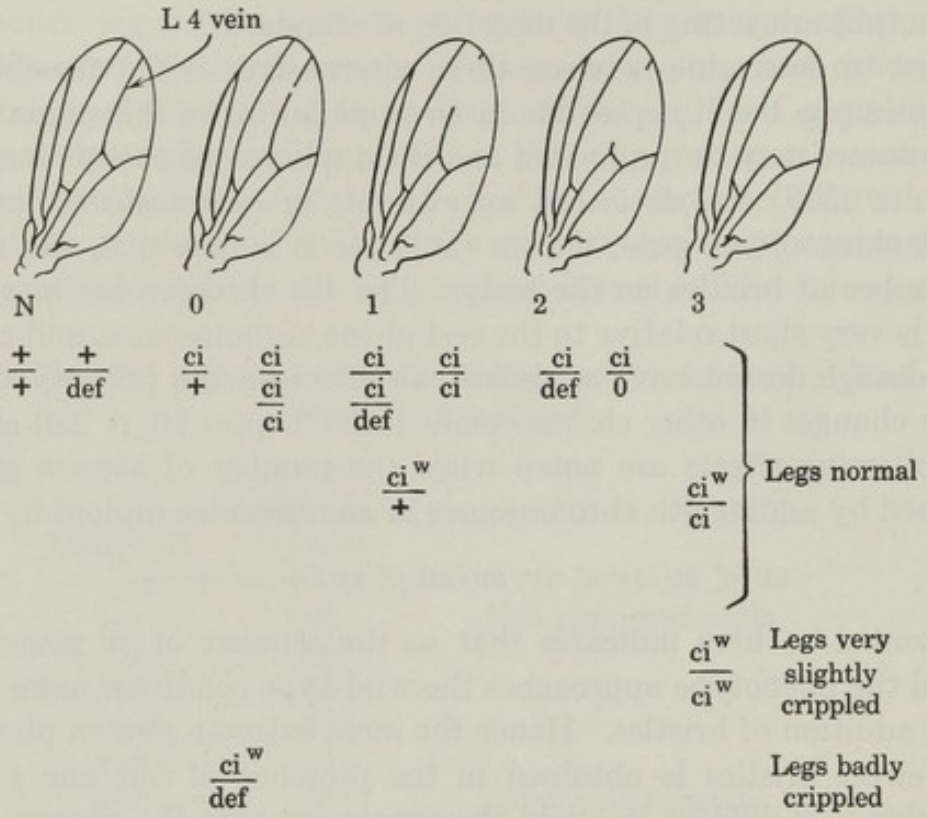


Fig. 67. The effects of the genes *ci*+, *ci*, and *ci*^{w} on the L4 vein and legs of *Drosophila melanogaster*. The deficiency is *def M*–4. It is a lethal when homozygous and may include loci other than *ci*; *o* indicates the absence of one of the fourth chromosomes. After Stern (606).

aberrantly as heterozygotes with other alleles. The *cubitus interruptus* (*ci*) allelic series in *D. melanogaster* located on chromosome 4 includes several good examples of this phenomenon (605, 606, 608). The mutant alleles in this series cause an interruption of the fourth vein (L4) in the wings (Fig. 67). The phenotypic expression is modified by the temperature with the most extreme mutant effect expressed at low temperatures around 14°C. In the experiments to be described, the degree of mutant expression was determined by the extent of interruption of the vein and the penetrance (the percentage of individuals observed of a particular genotype which show a mutant phenotype). All gradations between the extremes of intact fourth vein and its

complete absence from the cross vein to the tip of the wing occur as a result of substituting mutant alleles in different combinations. For convenience in measurement of gene effects the four arbitrary standards illustrated in Fig. 67 were defined, and mutant types were grouped in classes around these.

The effects of the three alleles, ci^+, *ci*, and ci^w, are as follows. The allele, *ci*, gives a more complete vein with increasing dosage, $+/+ > ci/ci/ci > ci/ci > ci/o$. This is precisely what one would expect from a hypomorph, but here the similarity ends, for, when *ci* is in combination with its + allele, a greater deviation from the wild type results than is produced by + heterozygous with a deficiency which includes the *ci* locus:

$$+/+ > +/def > ci/+$$

Thus although both *ci* and + act positively toward the production of an intact vein, + is haplo-insufficient, and *ci* "competes" with + when they are in combination. A more extreme example is provided by the ci^w allele (*cubitus interruptus* of Wallace) which is partially dominant over +, and causes *abnormal* venation with increasing dosage:

$$+/+ > +/def > ci^w/def > ci^w/+ > ci^w/ci^w$$

From this it would appear that ci^w is acting away from type effect, and "competes" strongly with the + allele so that the vein is even more reduced in $+/ci^w$ than in ci^w/def flies. A similar competition is shown in ci/ci^w flies. The subtractive effect of ci^w on type is accompanied by some capacity to produce type since one ci^w approaches normality. However, the other genes in the genome with effects on this character must not be overlooked for the indications are that the remainder of the deficient 4th chromosome is active in producing type effect too, in both *ci* and ci^w flies, as is shown in Fig. 67.

Since the *ci* alleles show an inhibition effect on type manifested particularly in compounds with + and one another, they may be described as *antimorphs* (443). The diagnostic character which distinguishes antimorphs from hypomorphs is a reduced capacity to produce a wild phenotype which is further impaired in heterozygotes with other alleles. There is an interaction of a competitive type indicated between alleles, resulting in a *subtractive* effect. Hypomorphs show a cumulative action toward wild type, whether homozygous or heterozygous, resulting in an *additive* effect.

Besides interrupting the wing vein, ci^w produces a crippled condition of the legs which in extreme cases may be so serious as to prevent the

emergence of the adult from the pupa case. The most extreme crippling results in ci^w/def flies which it will be recalled have almost normal wings. The other combinations of ci^w have the following relative phenotypic effects on the legs:

$$ci^w/def < ci^w/ci^w < ci^w/+ = +/+$$

The + allele is thus completely dominant to ci^w with respect to leg development, and since two ci^w produce more type effect than one ci^w, this allele is a hypomorph with respect to leg development at the same time that it is an antimorph with respect to wing development.

The *A* series of alleles in maize is another group which has been extensively investigated regarding allelic interactions of an antimorphic type. The genes in this series have some control over the production of anthocyans, anthoxanthins, and probably other pigments in the corn plant proper, as well as in the aleurone and pericarp of the kernels. At least three different types of pigments are affected by substitutions at the *A* locus: purple anthocyans which include the cyanidin derivative, chrysanthemin, yellow brown anthoxanthins including isoquercitin, and a red pigment in the pericarp, of whose chemical nature there seems to be no adequate information (535, 347). In general, when the purple and red pigments are absent, brown pigment replaces them in the pericarp and plant parts, but not in the aleurone which, in the genotypes to be described here, is either purple or colorless.

In Table 33 there are listed some of the known *A* alleles and the

Table 33. Effect of Substitutions at the *A* Locus in Maize *

Given for plants of the residual genotype *Pl B*.

	Phenotype of Heterozygote with *a*			Cumulative Effect with Increase in Dosage	"Competition" in Compounds with A^{br}
Allele	Plant	Aleurone	Pericarp †		
A-st	Purple	Purple	Red	0	–
A^b	Dil. purple	Purple	Dominant brown	0	–
A^{rb}	Dil. purple	Purple	Recessive red-brown	0	–
A^{br}	Dil. purple	Purple	Recessive brown	+	–
A^w	Pale ++	Pale ++	Recessive brown	+	0
A^d-41	Pale +	Pale +	Dominant brown	0	+
a^p	Pale	Pale	Dominant brown	0	+
a	Brown	Colorless	Recessive brown	0	0

* From Laughnan (346).

† Pericarp dominance relationships are cited for combinations with *A*-st.

phenotypes reported by Laughnan (346) resulting from the heterozygotes with the amorph *a* within the series. The standard allele *A*-st is dominant over all other alleles with respect to plant and aleurone pigmentation, but several alleles, A^b, A^d-41 and a^p produce a dominant brown pericarp in heterozygotes with *A*-st and A^{rb}. Considering only the plant and aleurone anthocyan color, the *A* alleles would appear to correspond to the guinea pig *C* series discussed previously, in so far as there is represented an amorph, a dominant standard producing full anthocyan, and a series of intermediates which answer the description of hypomorphs. Deviations from this simple pattern become evident, however, when the pericarp phenotypes and the results of interaction in heterozygotes are examined. Since A^{br} and A^w give cumulative effects with increasing dosage, they may be considered as hypomorphs. On the other hand, A^b, A^d-41, and a^p act contrary to the standard type effect by producing a dominant brown pigmentation in the pericarp and showing competition in compounds with other alleles such as A^{br}. For example, with respect to purple pigment:

$$A^{br}/a > A^{br}/a^p$$

But in the pericarp more brown pigment is produced by A^{br}/a^p than by A^{br}/a. Thus a^p acts in the direction of standard type by producing purple pigment, although not cumulatively, since $a^p/a^p = a^p/a$, but competes with purple pigment formation by other alleles. Further, it shows a positive action toward the production of brown pigment, and there is no competition evident with respect to brown in compounds with other alleles such as A^{br}. Like the ci^w gene in *Drosophila*, a^p, A^d-41 and A^b may be considered antimorphs with respect to one character, purple pigment formation, but toward another character, brown pigmentation, they appear as hypomorphs or complete dominants. Laughnan (346) has interpreted these results to mean that A^d-41, A^b and a^p control the synthesis of both anthocyans and brown pigment while the more typical hypomorphs affect anthocyan pigmentation only.

It is highly probable that the antimorphic type of effect is a relatively common phenomenon. Many hypomorphs may indeed be antimorphs to a slight degree not easily discernible, and every multiple allelic series can be expected to have some alleles which act as antimorphs.

While most mutant genes fall into one or more of the three categories, amorph, hypomorph or antimorph, when their effects on the phenotype are compared with their alleles, there are some which defy classification

in any one of these three groups. For example, a number of dominant mutant genes in *D. melanogaster* such as *Hairy wing, Bristle, Lyra,* and *Dicheate* produce a phenotype which is completely dominant to the wild type. Thus in the case of *Hairy wing* (443):

$$Hw/Hw > Hw/+ = Hw$$

If a duplication including the Hw^+ allele is added to any one of these three, there is no diminution in the extra number of bristles produced by *Hw*. Thus,

$$Hw/Hw/+ = Hw/Hw$$

$$Hw/+/+ = Hw/+$$

The normal + allele has no effect on the *Hw* phenotype, and therefore, relative to *Hw*, it is an amorph. Muller (443) has described *Hw* and others like it as *neomorphs* with the intention of implying that they have taken on an entirely new function from that previously carried out by the normal. This decision is based on the assumption that if there is no competition or synergistic action shown in heterozygotes, the two alleles must be unrelated in their function.

The absence of any indication of interaction between alleles is also evident in the inheritance of antigenic specificity. It will be recalled (Chapter 4) that heterozygotes consisting of two alleles, each controlling the production of a different antigen, show no dominance of one type over the other, although less of each type may be produced in heterozygotes. In the sense that each allele is "neutral" they may be considered as neomorphs. The neomorph concept may be further extended to the relationships existing between the incompatibility alleles in plants which show an independence of action comparable to that found among genes controlling antigenic specificity, although it has been shown by D. Lewis (367) that interactions between certain of these may be made evident in diploid pollen derived from tetraploids.

Biochemical explanations of the action of the various types of alleles discussed above may be made, if it is assumed that the effect on the phenotype by substitution of alleles can be extrapolated to the level of primary gene function. Hypomorphic alleles may then be assumed to produce a primary gene product directly involved in the production of type effect, which is only quantitatively different from the gene product of the normal, dominant allele, but limited in amount so that full type effect does not result. The amorphs may be assumed to pro-

duce no gene product or an amount so small as to have no effect on the phenotype. The neomorphs and antimorphs present a special problem which can best be resolved by the assumption of qualitatively different gene products being formed. Further consideration is given to these hypotheses in the following sections.

2. Dominance

In the usual allelic series one allele will exhibit complete dominance over all others such that in heterozygotes with hypomorphs, amorphs, or deficiencies it will produce the same phenotype as when homozygous. If the dosage of this "haplo sufficient" allele is increased beyond the diplo condition there will usually be no change in the phenotype. Its complete dominance will be weakened only by certain combinations with antimorphs, or reduced to zero in the presence of neomorphs. Between the extremes of complete dominance and the assumed neutrality of neomorphs, there is a range of intermediate conditions of incomplete dominance exhibited by hypomorphs and antimorphs in heterozygotes. The two phenomena, complete and incomplete dominance appear to differ only in degree, and it will be on this assumption that they will be discussed here.

It is a relatively simple matter to make a model to explain dominance in terms of quantitative gene action. If it be assumed that the lower alleles are hypomorphs or amorphs, the explanation becomes clearly one of degree of activity toward the production of the normal phenotype. The dominant allele is not only the most active, but it is overactive in the sense that when present once it produces sufficient or more than sufficient gene product to convert some substrate to a product necessary for the production of the normal phenotype. Any increase in its dosage may be expected to provide for more gene product, but since limits are set on the amount of product that may be active in producing type effect in the cellular environment, there will be no change in phenotype. Therefore, excess gene product will be ineffective. If the hypomorphic alleles produce less gene product than the dominant even when homozygous, they will obviously act as recessives, and when heterozygous with other hypomorphs they will be expected to show intermediate effects with no dominance of one allele over another. A diagram of this model is presented in Fig. 68. The quantitative data given for the guinea pig *C* series are in rough agreement with this interpretation, as are the phenotypic expressions of heterozygous combinations of many other allelic series. The threshold

effect which prevents further change in phenotype with increase in gene product concentration may be explained in terms of an assumed relationship between enzyme and substrate concentration, an explanation developed by Wright (709, 710).

It will be recalled from Chapter 6 in the discussion on enzyme kinetics that under certain conditions of enzyme excess relative to substrate a further increase in enzyme concentration will have little or no effect on the rate of formation of the product. (See Fig. 33*ab*.) If it is assumed that the dominant gene when present once allows for the production of a concentration of enzyme which is always in great excess of its substrate, then a further increase in enzyme (by increasing the gene dosage perhaps) will not be expected to increase the rate of product formation. The only factor which theoretically will affect the rate of product formation under these circumstances is a change in substrate formation. Application of enzyme kinetic theory can also be made to explain the results with hypomorphs. If it is assumed that a hypomorph allows for the production of such a small quantity of enzyme that the substrate concentration is in relative excess (see Fig. 68), then any change in enzyme concentration resulting from change in gene dosage will be expected to affect the rate of the product formation and hence the phenotype.

The concept that dominance of a gene is a manifestation of its superior activity as compared to its recessive alleles has its roots in the presence-absence hypothesis. There have merely been added more recently the supplementary observations that intermediate alleles, hypomorphs, exist which, as expected, show intermediate degrees of dominance.

While it is relatively simple to fabricate a model to explain dominance in cases where the alleles all seem to have a positive effect toward a specific phenotype, or are completely inactive with respect to the phenotype, difficulties are encountered in explaining dominance of antimorphs. However, if it is assumed that the alleles of a series may differ qualitatively as well as quantitatively with respect to their primary effect, an explanation readily becomes available (709, 710). By qualitative difference is meant a changed gene product (an enzyme, for example) which would convert the substrate to a product incapable of producing the normal phenotype to the same degree as that produced by products of genes which differ from the standard only quantitatively. (See Fig. 69.) The result of a qualitatively different gene product would therefore be "competition" for available substrate in the company of the more efficient hypomorphs; if the antimorphic gene product

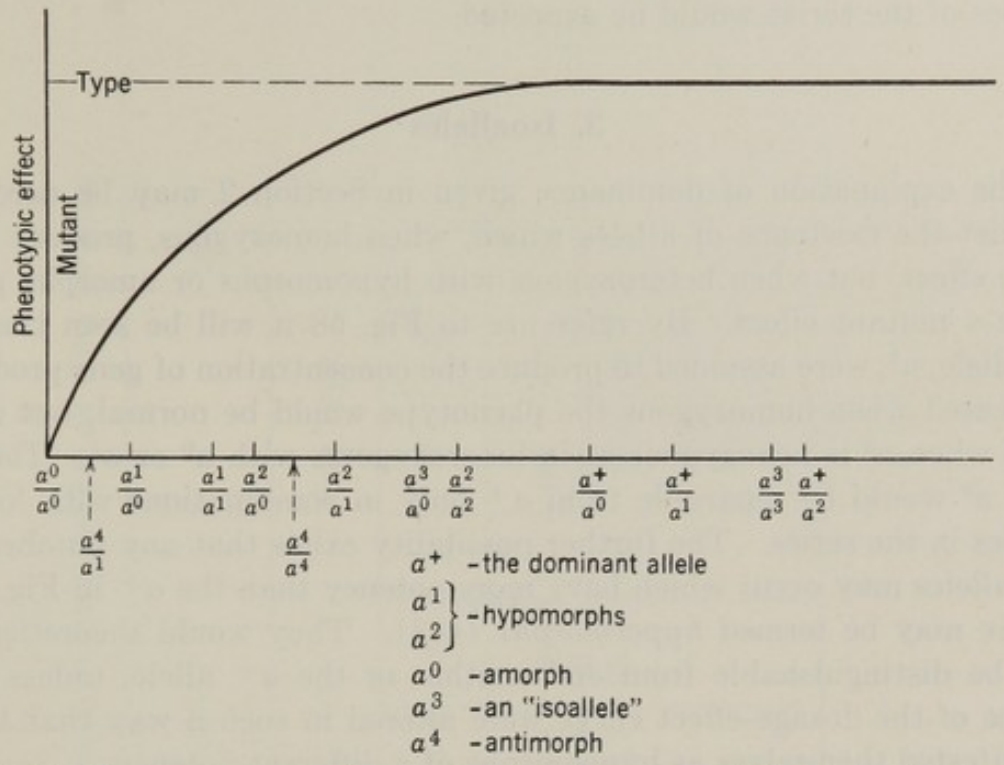


Fig. 68. Dosage in terms of concentration of gene product and its effect on the phenotype. A graph illustrating the hypothesis that an increase in the number of hypomorphic genes causes an increase in gene product with a concomitant effect on the phenotype. Any increase of gene product beyond a certain threshold value has no further effect on the phenotype for reasons discussed in the text.

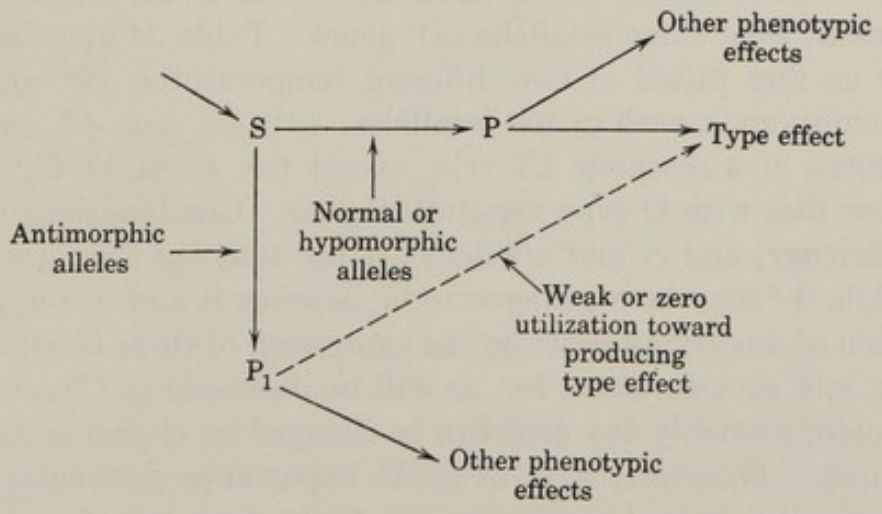


Fig. 69. A possible explanation for the mode of action of antimorphic genes.

is sufficiently potent, dominance over the hypomorphs and normal alleles of the series would be expected.

3. Isoalleles

The explanation of dominance given in Section 2 may be used to predict the existence of alleles which, when homozygous, produce full type effect, but when heterozygous with hypomorphs or amorphs produce a mutant effect. By reference to Fig. 68 it will be seen that if an allele, a^3, were assumed to produce the concentration of gene product indicated when homozygous the phenotype would be normal, but mutant when a^3 is hemizygous or in heterozygotes with a^0 or a^1. Therefore a^3 would be separable from a^+ only in combinations with lower alleles in the series. The further possibility exists that any number of a^+ alleles may occur which have more potency than the a^+ in Fig. 68. These may be termed *hypermorphs* (443). They would theoretically not be distinguishable from one another or the a^+ allele, unless the shape of the dosage-effect curve were altered in such a way that they manifested themselves as hypomorphs of a different potency.

Actual examples which fit the description of these hypothetical situations exist in what are known as *isoalleles*. These are so named because they are to be distinguished from one another, phenotypically, only when in certain heterozygous combinations, or when the gene modifier background or the external environment is changed. The *ci* series in *D. melanogaster* has been shown by Stern and Schaeffer (608) to include at least three isoallelic ci^+ genes. Table 34 gives the effect of these on flies raised at two different temperatures, 26° and 14°C. When homozygous, each of the isoalleles, $+^{ci}$, $+^2$, and $+^3$, causes the development of a complete L4 vein, except for $+^3$ at 14°C, in which case a few flies with O type venation appear. Combinations with the M-4 deficiency, and *ci* and ci^w demonstrate that the "weakest" allele is $+^3$ while $+^2$ may be considered to lie between it and $+^{ci}$ in potency. The action of modifying genes on the expression of these isoalleles must be taken into consideration, for, as will be discussed in Chapter 9, the expression of probably any gene can be changed by changing its genetic background. Therefore, it is of great importance particularly when investigating isoalleles to make the genetic background of each as uniform as possible in order to insure that the relatively slight phenotypic differences which distinguish them are not due to different modifiers. In the *ci* case this was approached by making the stocks isogenic for the X, 2nd, and 3rd chromosomes, but not for the 4th on which the *ci*

Table 34.* The Effects of Three Isoalleles at the *ci* Locus in *D. melanogaster* on Wing Venation †

Temperature	14°C			26°C		Temperature	14°C				26°C				
Genotype	Wing Type					Genotype	Wing Type								
	N	0	1	*N*	0		*N*	0	1	2	*N*	0	1	2	3
$+^{ci}+^{c}$	100	0	0	100	0	$+^{ci}/ci$	96.3	3.3	0.4	0	100	0	0	0	0
$+^{2}+^{2}$	100	0	0	100	0	$+^{2}/ci$	98.6	1.4	0	0	100	0	0	0	0
$+^{3}/+^{3}$	97.5	2.5	0	100	0	$+^{3}/ci$	32.6	42.7	23.4	1.3	69.8	20.7	9.5	0	0
$+^{ci}/def$	98.3	1.0	0.7	100	0	$+^{ci}/c^{w}$					28.8	16.9	50.5	3.7	0.09
$+^{2}/def$	88	10.8	1.2	100	0	$+^{2}/c^{w}$					16.2	17.3	54.2	11.2	0.4
$+^{3}/def$	92.7	6.6	0.7	99.1	0.9	$+^{3}/c^{w}$					1.0	1.4	26.1	54.2	17.3

* From Stern and Schaeffer (608).
† Data for females only.

locus is found. There exists the possibility therefore that modifiers close to the *ci* locus are the cause of the difference in expression noted, and that really only one + allele is present. It can be said only that within the limits of discrimination set by the genetic techniques, there exist three isoalleles in the *ci* series. This qualification applies, of course, to any allele, even those not considered as isoalleles.

It is highly probable that every gene has the potentiality of mutating to a very large number of different states and thus being transformed into an amorph, or a complete + dominant, or an antimorph, neo-

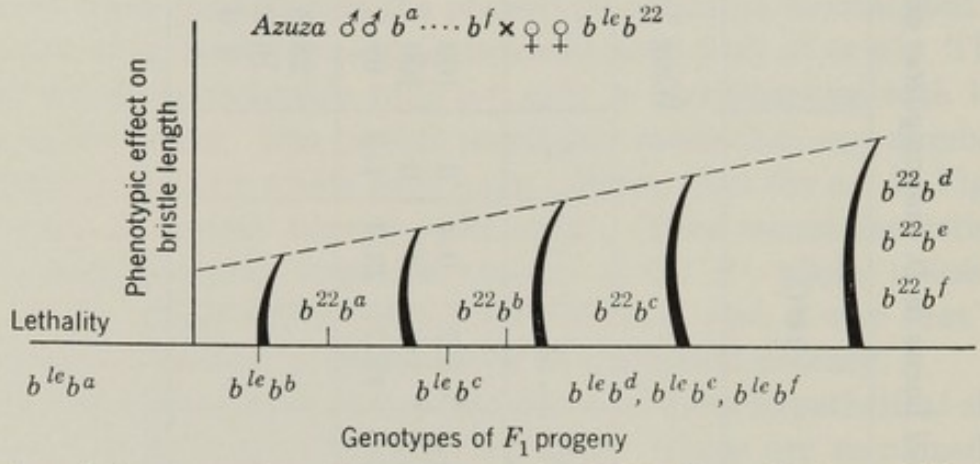


Fig. 70. A demonstration of isoalleles at the *bobbed* locus in *Drosophila hydei*. After Spencer (579).

morph, or hypomorph of a number of different potencies. The possibilities may be graded in a spectrum, from the amorphic condition on up with antimorphic and neomorphic deviations from type. Those close together in the spectrum may be considered as the isoalleles, and the differences in phenotypic expression of these must frequently be magnified by the alteration of the gene's background in order for them to be recognized. On the other hand, relative position in the spectrum may be so dependent upon external factors that members of isoallelic groups may appear to exchange positions, depending on the controlling circumstances.

A glimpse into the great complexity of the actual situation is afforded by the results of Spencer (579) obtained in his study of the isoalleles at the *bobbed* locus in *Drosophila hydei*. The *bobbed* genes in *hydei* reduce the size of the bristles just as does the *bobbed* gene previously described for *melanogaster*. Spencer found that the *bobbed* alleles procured from different populations of *hydei* produce a complex series of *bobbed* phenotypes. It was recognized that the differences in pheno-

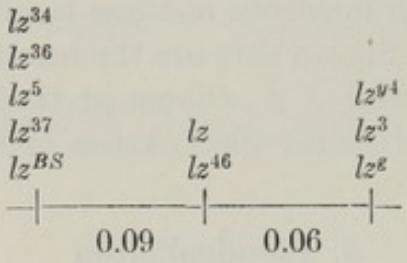
type could be due to a single type mutant allele acted upon by modifiers, but results obtained from the appropriate crosses indicated that a multiple allelic series is involved. The differences between alleles can presumably be exaggerated by modifiers, however. The alleles detected may be graded in a series according to phenotypic effect in homozygotes from extreme lethal types through to those which give normal phenotypes homozygous, but which produce a *bobbed* phenotype when heterozygous with intermediate *bobbed* alleles. An example which indicated the high incidence of these isoalleles in the population is presented in Fig. 70. Shown here are the results from a cross involving 12 phenotypically wild ♂ ♂, chosen at random, and crossed to a tester stock made up of Azusa (b^{22})/Azusa *bobbed lethal* (b^{le}) ♀ ♀.

4. Pseudoallelism

The possibility of the existence of genetic units with similar or related functions occurring closely linked together on the chromosomes has been postulated on many occasions (557, 3) to explain the peculiar phenotypic manifestations of some allelic series. Such "subgenes" or "pseudoalleles," as they are variously described, would be expected to be inherited as a unit with only extremely rare separation by crossing over. If mutation occurred in one of them, the "allele" so produced would be considered as allelic to the whole group of units rather than only to be the subunit from which it was derived, unless it could be separated from the group and recognized.

Until relatively recently the existence of such subunits, or the compound nature of certain loci, was in the realm of speculation. In 1940, however, Oliver (460) obtained a small number of unexpected wild-type *Drosophila melanogaster* from a cross involving two *lozenge* alleles, lz^g (*glossy*), and lz^s (*spectacle*), which could best be explained by assuming that the lozenge "locus" is compound. The lozenge alleles are recessive sex-linked genes which affect the phenotype in a number of ways: alterations in the amount of eye pigment, changes in the eye facets, and reduction of female fertility associated with the absence of spermathecae (462, 111). Heterozygotes between the mutant alleles show the lozenge effects on the phenotype, but the phenotype is not always intermediate. For example, in certain heterozygotes such as lz^g/lz^s, the flies are considerably more viable and fertile than the homozygotes, lg^g/lg^g or lz^s/lz^s (463). Thus a complementary effect is demonstrated rather than an intermediate quantitative effect which would be the expected result from the interaction of hypomorphs as discussed

in the previous sections. Oliver (461) found that, in a cross between heterozygous lozenge females lz^g/lz^s and either lz^g or lz^s males, some wild-type flies resulted which could be demonstrated to be genotypically lz^+. This could be explained on the basis of unequal crossing over as demonstrated for the *Bar* mutant by Sturtevant (625) which would mean that two *lz* alleles were caused to be located on one chromosome whereas the complementary type produced by the unequal cross-



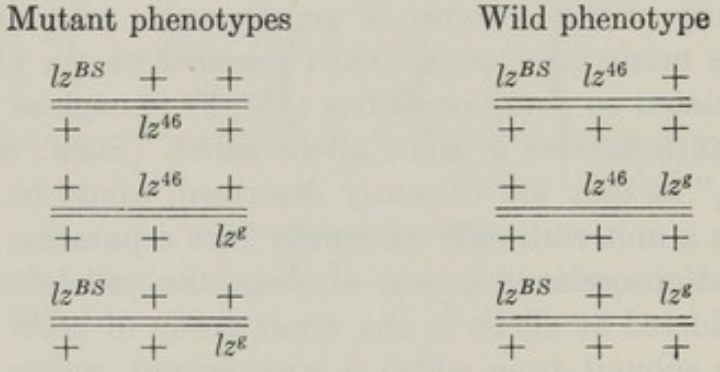


Fig. 71. The pseudoalleles of the lozenge series in *Drosophila melanogaster*. After Green and Green (230).

over would produce a normal phenotype. On the other hand it could be the result of equal crossing over between two closely linked genes which have similar effects on the phenotype. Thus if the "glossy" chromosome were genotypically $lz^g\ lz^s+$ and the "spectacle," $lz^g+\ lz^s$, and a cross over occurred between them, then the genotypes $lz^g\ lz^s$ and $lz^g+\ lz^s+$ would be expected (Fig. 71). The latter explanation seems to be the correct one in this example, as established by the work of Green and Green (230), who showed convincingly that the lozenge "gene" consists of at least three units or pseudoalleles which are separable by crossing over. Their map for these three loci is given in Fig. 71. They have located other alleles which appear to be "true" alleles at the indicated loci in the sense that they do not show crossing over except with those at other loci.

The question naturally arises at this juncture as to why $\frac{lz^{46} +}{+ lz^{g}}$ flies, for example, are mutant in phenotype where $\frac{lz^{46}\ lz^{g}}{+ +}$ flies are normal. (See Fig. 71 for further examples.) Since these are recessive genes, both genotypes would be expected to be normal. But other than the fact that heterozygotes with alleles on different chromosomes are more fecund and fertile than the homozygotes, lozenge phenotypic effects are expressed unaltered in the heterozygotes. The reasons for this peculiar type of "position effect" are not known. It has been noted not only in the lozenge series but in others in *Drosophila* such as the *Star-asteroid, stubble-stubbloid, bithorax-bithoraxoid,* and *white-apricot* pseudoallelic groups studied by Lewis (369, 371, 372, 373), and in *vermilion* by Green (228, 229). Obviously it is a phenomenon of some significance, and a complete understanding of it might well lead to a more precise idea of the mechanism of gene action, gene interactions, and of what constitutes mutation.

Lewis (372) has advanced a most ingenious explanation for the position effect phenomena of this type in connection with his studies on pseudoallelic series in *Drosophila.* He has found that one of the pseudoallelic groups studied by him consists of three adjacent loci, as illustrated in Fig. 72. The mutant genes at each locus have related

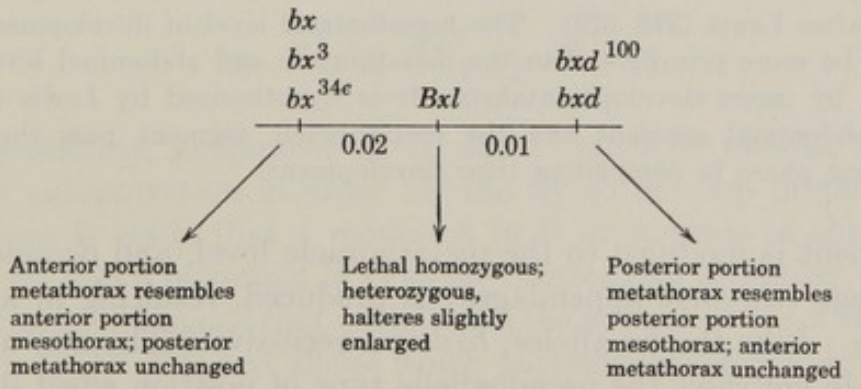


Fig. 72. The pseudoalleles of the bithorax-bithoraxoid series in *Drosophila melanogaster.* After Lewis (372).

but not identical effects on the phenotype. The bithorax alleles, *bx*, bx^{34e}, and bx^{3} are recessive genes which all cause a modification in different degrees of the metathoracic segment of the flies such that it appears to develop to the mesothoracic level. (See Fig. 73.) This is manifested in part by the haltere (a metathoracic appendage) being

modified to resemble a wing (a mesothoracic appendage), a phenotypic condition which results in varying degrees in flies carrying these genes.

The bithoraxoid gene, *bxd*, causes a somewhat similar but not identical modification of the metathorax to the mesothoracic level with similar accompanying changes in the haltere. In addition the first abdom-

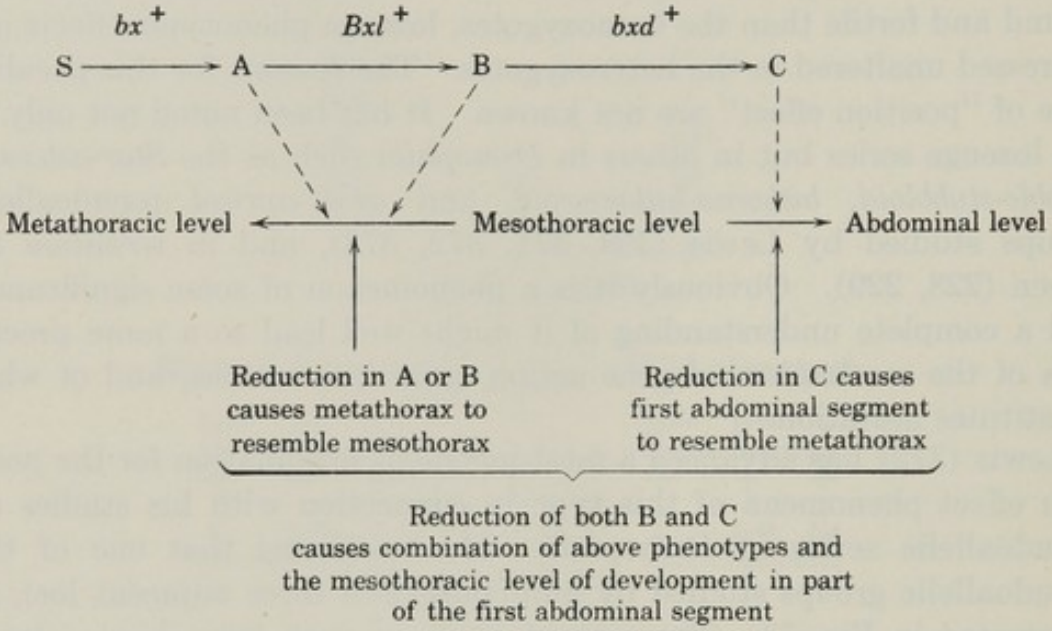


Fig. 73. A hypothetical scheme relating the genes *bx*+, *Bxl*+, and *bxd*+ to the development of the thorax and first abdominal segment in *Drosophila melanogaster*. After Lewis (370, 372). The mesothoracic level of development is considered to be more primitive than the metathoracic and abdominal levels of development by insect developmentalists. It is hypothesized by Lewis that both the first abdominal segment and the metathoracic segment pass through the mesothoracic phase in completing their development.

inal segment is modified to the metathoracic level, and occasionally a pair of legs (thoracic appendages) is produced, resulting in an eight-legged fly. Like the *bx* alleles, *bxd* is a recessive. The non-allelic *bx* and *bxd* do not show the pseudoallelic type of position effect described for lozenge, unless the most extreme alleles are combined. Thus phenotypically:

$$\frac{bx\ +\ +}{+\ +\ bxd} = \frac{bx\ +\ bxd}{+\ +\ +} = \frac{+\ +\ +}{+\ +\ +}$$

But with the extreme alleles bx^3 and bxd^{100} in the combination, $\frac{bx^3\ +\ +}{+\ +\ bxd^{100}}$, a slight bithoraxoid condition is manifested. When both

bx and *bxd* are present homozygous, the double mutant shows a combination of the bithoraxoid and bithorax phenotypes, with the further modification that the first abdominal segment also shows mesothoracic characteristics. Hence the effect of the *bx* gene may be said to extend to the first abdominal segment when in the presence of *bxd*, indicating a synergistic type of interaction on the part of the two genes.

Bithorax-like (*Bxl*)* is a dominant gene that is lethal homozygous. When present alone (+ *Bxl* +), it causes a slight enlargement of the haltere but has no other recognizable effects on the adult. Its functional relationship to *bx* and *bxd* is manifested particularly in the larvae. In the homozygous condition lethality is expressed in the larvae, and these show the characteristics demonstrated in the larval stages of *bx* + *bxd*/*bx* + *bxd* flies. Furthermore, *Bxl* acts as an allele of both *bx* and *bxd*. Thus, $\frac{bx\ +\ +}{+\ Bxl\ +}$ shows the bithorax phenotype, $\frac{+\ Bxl\ +}{+\ +\ bxd}$ flies are bithoraxoid, and $\frac{+\ Bxl\ +}{bx\ +\ bxd}$ are similar in phenotype to the homozygous *bx bxd* flies. This is a position effect of the pseudoallelic type, since $\frac{bx\ Bxl\ bxd}{+\ +\ +}$ flies are phenotypically similar to $\frac{+\ Bxl\ +}{+\ +\ +}$.

Lewis (372) proposes that since the three non-allelic genes are adjacent and have related effects on the phenotype, they may be involved in the control of series of reactions as follows:

$$S \xrightarrow{bx^+} A \xrightarrow{Bxl^+} B \xrightarrow{bxd^+} C$$

The hypothetical substances A, B, and C must be present at certain levels of concentration in order for the fly to develop properly. The assumption is made that a reduction in B or A leads to the bithorax phenotype, and a reduction in C to the bithoraxoid condition. The further assumption is made that mutation of the normal alleles of these genes results in the *bx*, *Bxl*, *bxd* alleles which partially or completely block the production of the indicated substances, resulting in the mesothoracic or metathoracic level of development in the metathorax and abdominal segment respectively. This is diagrammed in Fig. 73. The sequential scheme of function of the three genes is supported by a number of observations, among which may be noted the fact that when the bxd^+ gene is separated from bx^+ and Bxl^+ by rearrangement, an extreme bithoraxoid condition results. This seems to be a position effect due to a cytologically demonstrable transposition of a piece of chromosome to

* Also known as *Dominant bithorax* (bx^D) and *Ultrabithorax* (*Ubx*).

another part of the genome similar to the position effect discussed in Chapter 3 and is not a mutation of bxd^+ to bxd. Thus while bx^+ and Bxl^+ function normally when separated from bxd^+ (since no bithorax effect is noted) bxd^+ does not, and therefore its normal functioning is directly associated with close proximity to bx^+ and Bxl^+. In addition, it appears from duplications involving the normal alleles of bx and Bxl that Bxl does not directly function in producing the bithoraxoid condition but does control the production of something which must be present in sufficient quantity for the bxd^+ gene to function. If a duplication containing bx^+ and Bxl^+ is added to the homozygous Bxl genotype, $\frac{+\ Bxl\ +}{+\ Bxl\ +}$, the Bxl gene does not express itself as a lethal because of the presence of Bxl^+, but an extreme bithoraxoid phenotype is shown despite the absence of bxd. Hence it may be proposed that bxd^+ requires a substrate controlled by Bxl to produce the hypothetical C substance.

The sequential scheme fits all the above described data on the relations between these genes and their alleles (and others described by Lewis), provided one further assumption is made: the gene products A and B do not diffuse readily from the site of production on one chromosome to the corresponding site on the homologous chromosome. This would explain the position effect of pseudoalleles (and also the other types of position effect described previously) as shown in Table 35. Here the phenotypic effects of four possible combinations of bx^{34e} (a bx allele with slight mutant phenotypic effects), Bxl and bxd are described and interpreted according to the hypothesis presented above. In the first example the mutant alleles are all on one chromosome, and the required hypothetical substances A, B, and C are produced by the three normal alleles on the homologue. The slight reduction in A indicated by the sign, due to the presence of bx^{34e}, and the correspondingly more drastic reductions in B and C, are not great enough to reduce the total concentrations to a level where an extreme mutant phenotype is produced. Only B is reduced sufficiently to show an effect. In (2) a position effect is demonstrated resulting in a bithorax phenotype, since B is reduced in amount by blocks formed by bx^{34e} and Bxl. The A produced in the presence of $bx^{34e}+$ cannot diffuse to the corresponding homologue to be converted there to additional B in the presence of Bxl^+. It is assumed that the indicated reduction in C

Table 35. The Phenotypic Effects of Various Combinations of the Pseudoalleles bx^{34e}, *Bxl*, and *bxd* and Their Interpretation according to Lewis (372)

	Genotype	Phenotype	Interpretation on Basis of Sequential Arrangement and Function
(1)	$\frac{+\ +\ +}{bx^{34e}\ Bxl\ bxd}$	Enlarged haltere like $\frac{+\ Bxl\ +}{+\ +\ +}$; otherwise normal	$\overset{+}{\text{S}} \longrightarrow \overset{+}{\text{A}} \longrightarrow \overset{+}{\text{B}} \longrightarrow \text{C}$ / $\underset{bx^{34e}}{\text{S}} \longrightarrow \underset{Bxl}{<\text{A}} \longrightarrow \underset{bxd}{\ll\text{B}} \longrightarrow \ll\text{C}$
(2)	$\frac{+\ Bxl\ bxd}{bx^{34e}\ +\ +}$	Moderate bithorax phenotype	$\overset{+}{\text{S}} \longrightarrow \overset{Bxl}{\text{A}} \longrightarrow \overset{bxd}{\ll\text{B}} \longrightarrow \ll\text{C}$ / $\underset{bx^{34e}}{\text{S}} \longrightarrow \underset{+}{<\text{A}} \longrightarrow \underset{+}{<\text{B}} \longrightarrow <\text{C}$
(3)	$\frac{+\ +\ bxd}{bx^{34e}\ Bxl\ +}$	Extreme bithoraxoid, no bithorax condition	$\overset{+}{\text{S}} \longrightarrow \overset{+}{\text{A}} \longrightarrow \overset{bxd}{\text{B}} \longrightarrow \ll\text{C}$ / $\underset{bx^{34e}}{\text{S}} \longrightarrow \underset{Bxl}{<\text{A}} \longrightarrow \underset{+}{\ll\text{B}} \longrightarrow \ll\text{C}$
(4)	$\frac{bx^{34e}\ +\ bxd}{+\ Bxl\ +}$	Combination of bithorax and bithoraxoid and nearly lethal	$\overset{bx^{34e}}{\text{S}} \longrightarrow \overset{+}{<\text{A}} \longrightarrow \overset{bxd}{<\text{B}} \longrightarrow \ll\text{C}$ / $\underset{+}{\text{S}} \longrightarrow \underset{Bxl}{\text{A}} \longrightarrow \underset{+}{\ll\text{B}} \longrightarrow \ll\text{C}$

Note: < designates a slight reduction, and ≪ a more drastic reduction in the indicated substances.

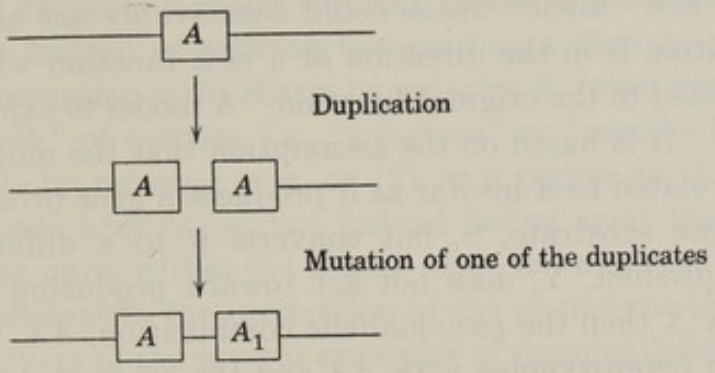
is not great enough to cause the appearance of the bithoraxoid phenotype. In the next two examples, however, it will be noted that C is expected to be greatly reduced according to the hypothesis, and an extreme bithoraxoid condition results. In (3) there is sufficient B to permit the normal bithorax condition, but in (4) both B and C are greatly reduced, and the combination effect results.

Although a large number of assumptions are required to construct a model to explain the bithorax-bithoraxoid data, it is evident that they are reasonable assumptions; and a working hypothesis which may be of considerable significance in directing the course of future research is gained thereby. It will be recalled that a number of enzyme systems are known to act as a unit in which each type of enzyme molecule is apparently in close proximity to the others of the system, as in the mitochondria. It may well be that groups of closely related genes may function in the organization of such enzyme systems, and that dis-

organization of the spatial arrangement of the enzymes in the systems may result from a change in the relative positions of the genes so that a position effect is manifested.

Numerous other examples are known of closely linked genes which affect related aspects of the phenotype when they mutate. In *Drosophila* alone some 50 such groups have been tabulated by Grüneberg (235) and Goldschmidt (209), and although none of them have been analyzed in the detail described for the *lozenge* and *bithorax* pseudoalleles, it is highly probable that many of them represent pseudoallelic series of the type described above. Possible examples of pseudoalleles have been found in certain wasps (687), in *Aspergillus* (491) and also in cotton (601, 602) and *Neurospora* (53, 195). Many multiple allelic series may represent cases of pseudoallelism. Indeed, the classic example of a multiple allelic series, the white alleles in *D. melanogaster* which control the production of eye pigment, is now known to include pseudoalleles. The distinguishable mutant alleles in this series number about 10 or more with the *white* (*w*) allele acting as an amorph giving no pigment in the eyes, and the remainder acting as hypomorphs or antimorphs and producing intermediate amounts of pigment. One of the intermediate alleles, *apricot* (w^a or *apr*), has been shown to cross over with *w* so that flies of the genotypes *apr w*, *apr* w^+, $+^{apr}w$, etc., are now known to exist (373). The other "alleles" of the series have not been located with certainty, but it is now evident that the *white* series contains at least two loci. It will be recalled that in Chapter 4 one of the explanations for the multiplicity of inherited antigenic types prevalent in the domestic cattle population was that they may be produced, in part, by a series of closely linked genes. Proof of this would be, of course, contingent upon demonstrating crossing over between the assumed genetic units. Pseudoallelism is particularly indicated for those allelic series described in section 1, the members of which show unexpected interactions in heterozygotes, such as the *A* alleles in maize and the *ci* alleles in *Drosophila,* which were described as *antimorphs.* Indeed Laughnan (348) has obtained considerable evidence that indicates a compound nature for the *A* locus in maize. The spontaneous origin of A^d alleles from A^b in maize is associated with crossing over at the *A* locus. Hence, A^b may consist of two or more pseudoalleles. This observation is of considerable significance in establishing an explanation for the action of antimorphs in this and other allelic series like it. And it can best be understood by considering the possible reasons for the existence of pseudoalleles and the theoretical evolutionary consequences of their existence.

It appears certain that the physical association of genes of like function on the chromosomes is not fortuitous, for the number of examples now known is very large, if one considers the available data from all sources. Therefore, it is logical to assume that pseudoalleles arise by



If the pseudo allele A_1, takes on a new function as a result of the mutation which is related to the function of A, then two alternative schemes are suggested:

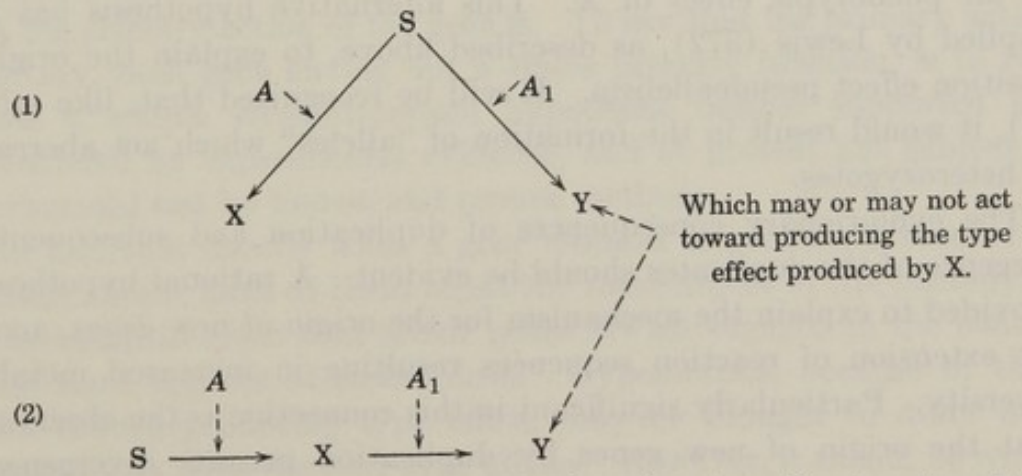


Fig. 74. Hypotheses to explain the origin of antimorphs. Based on hypotheses presented by Lewis (372).

duplications or repeats wherein a gene is duplicated and the duplicate retained on the same chromosome (Fig. 74). One possible mechanism for the origin of this type of duplication of a small segment is unequal crossing over, as has been demonstrated in *Drosophila* by Bridges (72). There is cytological proof that such duplications do occur (71, 409), and analysis of *Drosophila* salivary gland chromosomes show that the *star-asteroid, stubble-stubbleoid* and *bithorax-bithoraxoid* pseudoallelic groups are associated with what appear to be duplications of the chromosome material (372).

Once such a duplication has occurred, it may be manifested in the phenotype because of a double dose of a gene being present, and the duplicate will be inherited as an allele of the original condition. Such a phenomenon seems to have taken place in the origin of the *Bar* mutants of *Drosophila* (625). Furthermore, one of the duplicates may mutate and produce a new "allele" which could conceivably act as an antimorph if the mutation is in the direction of a new function which may or may not be related to the original function. A model to explain this is given in Fig. 74. It is based on the assumption that the mutant gene A has a function related to *A* insofar as it produces a gene product which acts on the same substrate, S, but converts it to a different product. If the new product, Y, does not act toward producing the phenotype produced by X then the pseudoallelic combination, *AA*, will act as an antimorph in heterozygotes with *AA* and the original *A*, for there will be competition for the available S. An alternative condition is shown in Fig. 74 in which it is postulated that *A* converts X to a new product Y which has a new phenotypic effect which may be related to the phenotypic effect of X. This alternative hypothesis has been applied by Lewis (372), as described above, to explain the origin of position effect pseudoallelism. It will be recognized that, like scheme (1), it would result in the formation of "alleles" which act aberrantly in heterozygotes.

The evolutionary consequences of duplication and subsequent divergence of the duplicates should be evident. A rational hypothesis is provided to explain the mechanism for the origin of new genes, and for the extension of reaction sequences resulting in increased metabolic diversity. Particularly significant in this connection is the observation that the origin of new genes by duplication permits divergence of function by one of them while the other persists and maintains the old function (601).

5. A Reexamination of Some Definitions

Genes have been variously described as (1) units not reduced by crossing over, (2) units of mutation, and (3) units of function. It is germane to reexamine these definitions in connection with a discussion of alleles, for as indicated previously a gene is recognized only when it exists in at least two allelic forms. What is meant by alleles, therefore, decides to a very large extent what is meant by the more general term, gene.

So long as the units of function as determined by mutation corresponded to units not reduced by crossing over, the definitions given above were valid descriptions of the experimental observations. However, the proof that crossing over occurs in those chromosomal regions formerly described as genes, because they fulfilled these definitions, now requires a closer scrutiny of the whole matter. It would appear that instead of viewing the gene as a discrete physical entity on the chromosome with sharp boundaries it must now be viewed (1) as a "block" of entities, i.e., "subgenes" or "pseudoalleles" which are separable by crossing over, or (2) as a region on the chromosome with no definite boundaries, but instead fading away into the next gene. That these units within the blocks seem to be related in function and interdependent in the production of the phenotype is well supported by the analyses of the several examples of pseudoallelism discussed above.

When the gene is defined as a unit of function, it means that as a result of its mutation some consistent effect is produced on the phenotype which is related to the gene's action. How directly related the gene's action is to the phenotype is a moot question, since no one knows what the primary action of the gene is. To say that the primary action is unitary, each gene having but a *single* primary function, is to formulate a simple straightforward hypothesis neither supported nor contradicted by experimental evidence, and at present not subject to experimental test by known and proven methods.

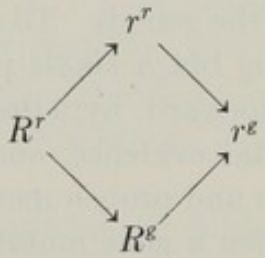
The fact that usually when a gene mutates to produce a number of different alleles, most of them affect the same aspect of the phenotype, can be taken to mean that allelic genes are all engaged in the control of the same aspects of metabolism. Hypomorphs, because of their action toward producing type effect, may be thought to differ only quantitatively from the wild-type alleles. However, it should be recognized that a quantitative change in phenotype, such as a change in the amount of pigment, need not be the result of a simple quantitative change in the gene's action. A gene may, like an enzyme, change its activity by undergoing a qualitative change such as in its surface configuration. In so doing its specificity may be altered, but the change in phenotype may only be quantitative. Antimorphic effects may, of course, be the result of such changes in specificity too, in which a qualitative change in phenotype is produced.

It is also in the realm of possibility that each gene has a number of different active centers any one of which may change (mutate) independently of the others. This would mean that as a unit of mutation it is a complex unit with a number of related specificities which might

be changed by mutation, but the whole functioning, nonetheless, as a unit. Such a possibility can be visualized in connection with pseudo-alleles, and receives experimental support from observations like those of Stadler (596) on the *R* series of alleles in maize. The alleles at the *R* locus control the production of purple anthocyan pigment in both the endosperm of the kernels and the plant (leaves, etc.). The many known alleles may be classified into four groups, dependent upon whether they affect the seed or plant pigmentation or both, as follows:

R^r—purple seed, purple plant
R^g—purple seed
r^r—purple plant
r^g—no anthocyan in seed or plant.

In a very extensive series of experiments Stadler (596) was able to show that three of these alleles mutate spontaneously at rates dependent upon the residual genotype (see p. 47) according to the following pattern:



Mutations of R^r to r^r (plant pigment only) and R^g (seed pigment only) occur independently. The gene R^r appears therefore to consist of two elements with partially independent action and independent mutation. Since no crossing over has been observed between these elements, there can be no decision made regarding their relation to one another other than to suggest that *R* is a "compound" gene (596) consisting of two related elements. Such a conclusion, however, makes it clear that the definition of a gene as a unit of mutation is a vague one and open to considerable qualification as to what is meant.

CHAPTER 10

Interactions of Non-Allelic Genes

Whenever breeding tests demonstrate that the inheritance of a particular character is dependent upon more than one known gene, the phenotype is defined as a manifestation of gene interaction. This definition holds whether the genes are allelic or non-allelic. Since allelic interactions have already been discussed, it will be the purpose here to consider non-allelic interactions and the diagnostic methods used to distinguish between the various types.

To appreciate the difference between the inheritance of a character due to a single gene or a pair of alleles (monogenic inheritance), and one due to two or more non-allelic genes (digenic, etc., inheritance), it is necessary to understand the mechanics of certain types of breeding tests and their consequences. For example, consider the inheritance of white eyes in a certain strain of *Drosophila melanogaster* which breeds true for this character. The mutant white-eyed strain is crossed, following the usual procedure, to a wild-type strain known to be homozygous for the normal red eye color. The resultant F_1 is composed only of flies with red eyes, showing that the mutant character is recessive to normal. The F_1 hybrids are then inbred and the F_2 phenotypic ratio determined to be 9 wild type:3 brown-eyed:3 bright red-eyed:1 white-eyed. This is the ratio expected from 2 pairs of genes segregating independently, and demonstrates that white eyes are determined here by the simultaneous presence of two mutant genes. Further test crosses of the mutant types to genotypically known mutant strains make it possible to determine the identity of the mutant genes involved. It will be assumed in this example that they are *scarlet* (*st*) and *brown* (*bw*). The genotypic formula of the white-eyed strain may then be written as *st/st, bw/bw,* and the original test crosses described with the appropriate symbols as shown Fig. 75.

The gene *st* has been discussed previously (p. 214) as blocking the

formation of the brown pigment component of the normal colored eye. It is also known that the gene *bw* blocks the formation of the red and yellow eye pigments which together with the brown pigment give the normal eye pigmentation of the wild-type fly. With this information the F_2 results become intelligible. Wild-type flies have both components due to the presence of both st^+ and bw^+, brown-eyed flies are st^+ *bw*, and bright red-eyed flies have no brown pigment, being *st* bw^+.

Parents $\frac{st^+}{st^+}\frac{bw^+}{bw^+} \times \frac{st}{st}\frac{bw}{bw}$

Wild type White eyes

F_1 All $\frac{st^+}{st}\frac{bw^+}{bw}$

F_2 Ratio of:

9 Wild type: $\frac{st^+}{st}\frac{bw^+}{bw}$, $\frac{st}{st^+}\frac{bw^+}{bw^+}$, $\frac{st^+}{st^+}\frac{bw}{bw^+}$, $\frac{st^+}{st^+}\frac{bw^+}{bw^+}$: 3 Brown eye: $\frac{st}{st^+}\frac{bw}{bw}$, $\frac{st^+}{st^+}\frac{bw}{bw}$: 3 Bright red eye: $\frac{st}{st}\frac{bw^+}{bw^+}$, $\frac{st}{st}\frac{bw}{bw^+}$: 1 White eye: $\frac{st}{st}\frac{bw}{bw}$

Fig. 75. A demonstration of digenic inheritance involving the character white eye in *Drosophila melanogaster*.

With the failure of production of both pigments in *st bw* flies, white eyes result.

If *bw* and *st* were closely linked instead of being on separate chromosomes (the 2nd and 3rd respectively), the ratio obtained in the F_2 would have been different. The recombination classes *st* bw^+ and st^+ *bw* would be proportionately lower in number, and the ratio would approach 3 wild type:1 white-eyed which would be characteristic of monogenic inheritance. The closer the two genes are linked the closer would be the approach to the 3:1 ratio. Complete linkage, i.e., no crossing over between *st* and *bw*, would result in a simple 3:1 ratio, and the conclusion would be that only one gene was involved in producing white eyes. Thus, in another situation, if a white-eyed strain of the genotype *w*/*w* (see p. 226) is tested, the F_2 ratio will be 3 wild type:1 white-eyed with no intermediate eye colors among the progeny. The genotypic formula of the two white-eyed strains should be written $\frac{w}{w}\frac{st^+}{st^+}\frac{bw^+}{bw^+}$

for the monogenic strain, and $\frac{w^+}{w^+}\frac{st}{st}\frac{bw}{bw}$ for the digenic, to be complete. However, brevity dictates that only the mutant gene should be included in the genotypic formula, the wild-type residual genotype being assumed.

The mutants *w/w* and *bw/bw* have been found to produce the diffusible precursors to the formation of brown pigment (30). Since the

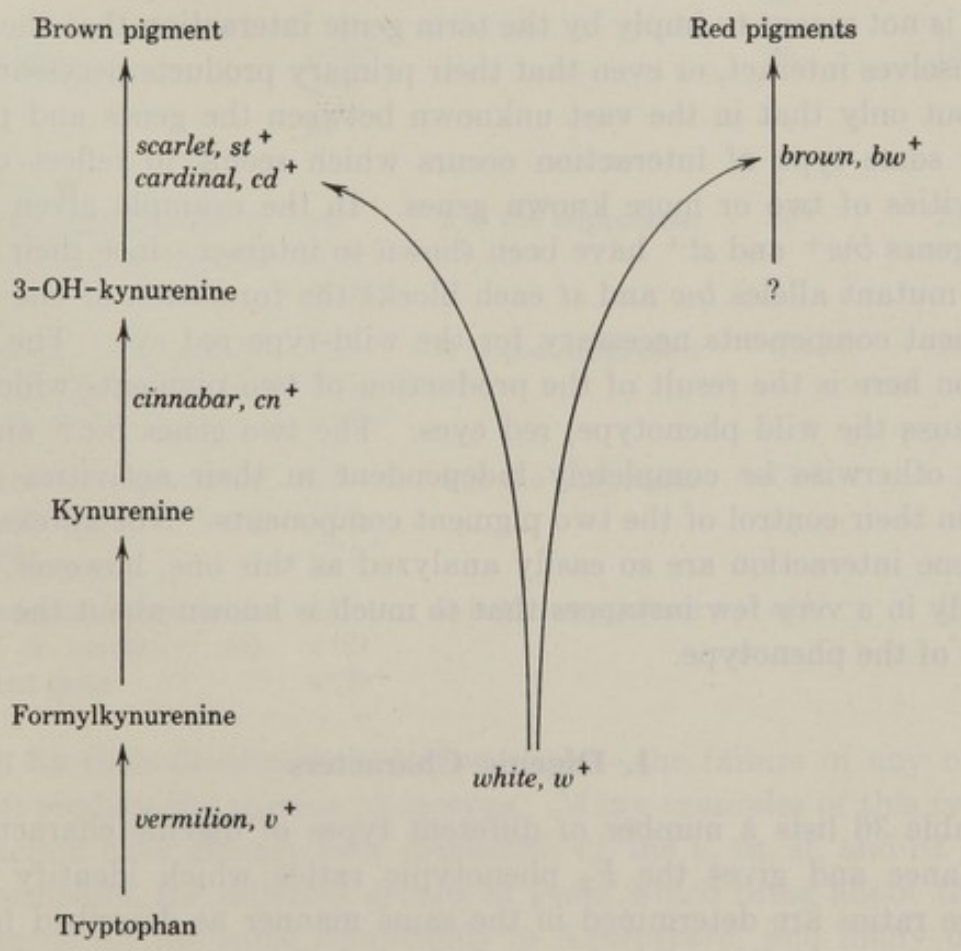


Fig. 76. The assumed relationship of the eye color genes in *Drosophila melanogaster*.

white gene blocks the formation of both pigment components and the *brown* gene only the red component, one may construct a metabolic map for the formation of *Drosophila* eye pigment using this information and that given on p. 215 with the result shown in Fig. 76. This interrelationship between several genes is probably one of the best examples of gene interaction known.

Essentially the same breeding test technique described for *st*, *bw* in *Drosophila* is used to distinguish between monogenic and digenic in-

heritance in haploids like *Neurospora,* but since there is no problem of heterozygosity with a dominant masking the expression of a recessive allele, the test crosses are even simpler. A cross of a mutant strain to a wild type should result in a ratio of 1 wild type:1 mutant in the F_1, if the mutant character is due to a single allelic difference. If the character is digenic, etc., there will be a deviation from this ratio such as 3:1 or 1:3. Examples of such deviations are discussed in the following sections.

It is not meant to imply by the term genic interaction that the genes themselves interact, or even that their primary products necessarily do so, but only that in the vast unknown between the genes and phenotype some type of interaction occurs which seems to reflect on the activities of two or more known genes. In the example given above the genes bw^+ and st^+ have been shown to interact, since their recessive mutant alleles *bw* and *st* each blocks the formation of one of the pigment components necessary for the wild-type red eye. The interaction here is the result of the production of two pigments which mix to cause the wild phenotype, red eyes. The two genes bw^+ and st^+ may otherwise be completely independent in their activities in the cell in their control of the two pigment components. Not all examples of gene interaction are so easily analyzed as this one, however, for it is only in a very few instances that so much is known about the chemistry of the phenotype.

1. Digenic Characters

Table 36 lists a number of different types of digenic character inheritance and gives the F_2 phenotypic ratios which identify them. These ratios are determined in the same manner as described for the *st, bw* example discussed above. Independent assortment of the gene pairs considered is assumed, and the cited ratios are obtained only under this condition. If there is linkage of the interacting genes, as is frequently the case, the ratio will be dependent upon the number of recombinations resulting from the crossing over which occurs.

Complementary Genes

One of the commonest types of gene interaction involves non-allelic genes which give the same mutant phenotype. They are called complementary genes or mimic genes. In order for the wild type or standard phenotype to be expressed, all complementary genes must be repre-

Table 36. Ratios Expected in the F_2 in Different Types of Digenic Character Inheritance in Diploids Assuming Independent Assortment

a and b, recessive mutant genes; a^+ and b^+, respective normal alleles; C and D, dominant mutant genes; C^+ and D^+, respective recessive normal alleles

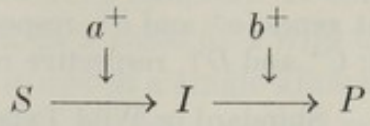
	Standard or Wild Type		Mutant	F_2 Ratio
Complementary	a^+b^+		ab a^+b ab^+	9:7
Duplicates	a^+b^+ a^+b ab^+		ab	15:1
Suppressors				
Recessive suppressor of a recessive mutant gene	ab a^+b a^+b^+	b is the suppressor	ab^+	13:3
Dominant suppressor of a dominant mutant gene	CD C^+D C^+D^+	D is the suppressor	CD^+	13:3
Recessive suppressor of a dominant mutant gene	Cb C^+b C^+b^+	b is the suppressor	Cb^+	7:9
Dominant suppressor of a recessive mutant gene	aD a^+D a^+D^+	D is the suppressor	aD^+	15:1

sented by their dominant wild-type alleles—the failure of any one or all will produce the mutant phenotype. Many examples of this type of interaction have already been discussed; cf. the *v*, *cn*, *st*, and *cd* genes in *Drosophila*, the different groups of genes which bring about the requirement for the same compounds in *Neurospora*, and many of the genes involved in anthocyan and anthoxanthin pigment formation in plants.

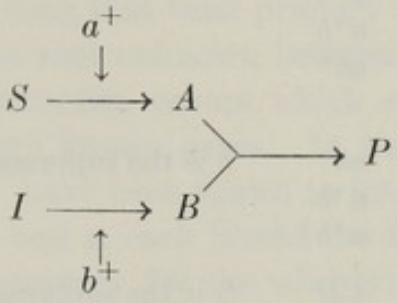
Two complementary genes segregating independently will give a phenotypic ratio of 9 wild type to 7 mutant, unless, as described for *st* and *bw*, single recessives can be distinguished phenotypically from one another and the double recessive with the result that a 9:3:3:1 ratio is obtained. In haploids such as *Neurospora* the result of a cross between two phenotypically similar mutant strains each carrying a different mutant gene will be a ratio of 1 wild type to 3 mutant.

The usual biochemical interpretation of the action of complementary genes is that they are involved in a reaction sequence. Thus, if *a* and

b are complementary in the wild type the following model of the action of the normal alleles could be made:



where *P* is required for the expression of the wild phenotype. If either *a* or *b* or both were present to block its production, the mutant phenotype would result. However, alternative explanations such as



can also be applied to many examples, and it is to be recognized that these models as well as those given below have heuristic value only.

Duplicate Genes

Duplicate genes have been found in a number of plants. These are recessive non-allelic genes which when present both together in the homozygous condition produce a digenic mutant phenotype. Duplicate genes are known in maize, especially among the genes affecting chlorophyll production (160), in tobacco (110), and wheat (555). Duplicate gene inheritance is recognized by a 15:1 ratio of wild type to mutant in the F_2, if there is independent assortment.

The relatively common occurrence of duplicate and even triplicate and quadruplicate genes in plants is probably the result of the fact that many plants, particularly the cultivated varieties used most often in genetic experiments, are hybrid polyploids. (See Fig. 5.) Origin from polyploids would be expected to result in the duplication of genes of like function, each parent contributing a member of a duplicate set as shown in Fig. 77. The phenomenon of two mutant genes being required to produce a mutant phenotype may then be the result of duplication of action rather than gene interaction. In animals polyploidy is an uncommon phenomenon which must have played a very minor role in evolution, and in view of this it is not surprising to find that duplicate genes of this type are practically unknown among them.

Although duplicate gene inheritance is strictly not an example of

gene interaction, it is evident that genes with related functions showing interactions may have arisen from duplicates during the evolutionary process (601, 602). Among the various species of cultivated cotton, for example, which are known to be amphidiploids, there are a number of peculiar types of inheritance of mutant characters best explained by assuming a divergence of function of originally duplicate genes.

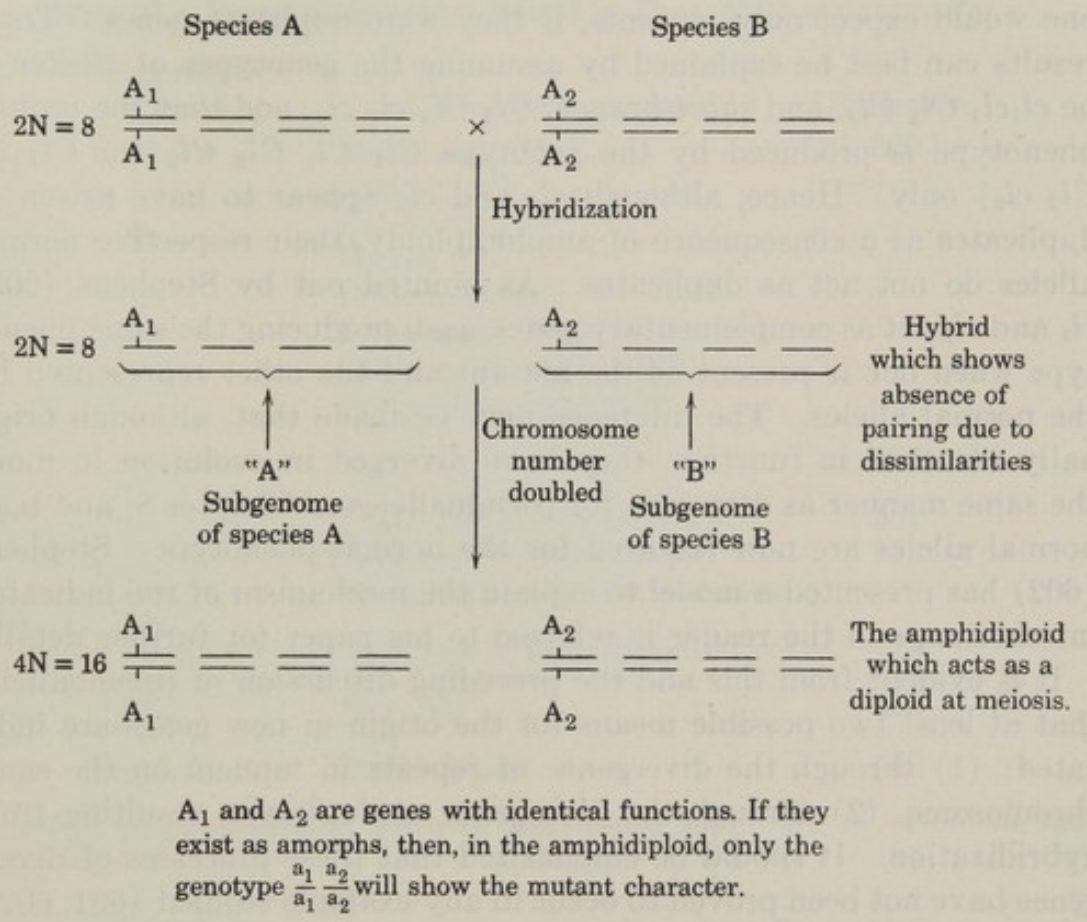


Fig. 77. The origin of an amphidiploid, tetraploid, by hybridization and doubling of the chromosome number.

The amphidiploid cotton species, *Gossypium hirsutum* and *G. barbadense* each have a mutant phenotype inherited as a single gene difference involving a shortening of the flower branches to produce flower clusters. The mutant is known as *cluster* in *hirsutum*, and *short branch* in *barbadense*. Cytologically, *hirsutum* and *barbadense* are similar. Both appear to have been derived originally by hybridization of a diploid species with 13 pairs of chromosomes of the "D" type and a diploid with 13 pairs of "A" chromosomes (598, 601). The amphidiploids, therefore, have a chromosome constitution of 26 A + 26 D and would be assumed to carry a number of duplicate sets of genes. This

assumption is in part supported by the presence of the *cluster* mutant gene in the D chromosome set or subgenome of *hirsutum* and *short branch* in the A subgenome of *barbadense*. However, the assumption that *cluster*, cl_1, is a true duplicate of *short branch*, cl_2, because they both produce the same phenotype is not borne out by the results of breeding experiments. If *cluster hirsutum*, $cl_1\ cl_1$, is crossed to *short branch barbadense*, cl_2cl_2, the F_1 progeny are all normal (563), whereas one would expect only mutants, if they were duplicate genes. These results can best be explained by assuming the genotypes of *cluster* to be $cl_1cl_1\ Cl_2\ Cl_2$, and *short branch*, $Cl_1\ Cl_1\ cl_2\ cl_2$, and that the normal phenotype is produced by the genotype $Cl_1\ Cl_1\ Cl_2\ Cl_2$ (or $Cl_1\ cl_1\ Cl_2\ cl_2$) only. Hence, although cl_1 and cl_2 appear to have arisen as duplicates as a consequence of amphidiploidy, their respective normal alleles do not act as duplicates. As pointed out by Stephens (602) cl_1 and cl_2 act as complementary genes, each producing the same phenotype when one is present as the mutant and the other represented by the normal alleles. The inference may be made that, although originally identical in function, they have diverged in evolution in much the same manner as discussed for pseudoalleles in Chapter 8, and both normal alleles are now required for the normal phenotype. Stephens (602) has presented a model to explain the mechanism of the indicated interaction, and the reader is referred to his paper for further details.

It is evident from this and the preceding discussion of pseudoalleles that at least two possible means for the origin of new genes are indicated: (1) through the divergence of repeats in tandem on the same chromosome, (2) through the divergence of duplicates resulting from hybridization. It should be emphasized that these processes of divergence have not been proven to occur in any example studied (601, 602). Nonetheless, an appreciation of the possible existence of such a mechanism and the study of the biochemical relationships of genes apparently so related may well lead to a better understanding of gene action and interaction in metabolism, for present-day genotypes are the result of long evolutionary development.

Suppressor Genes

The geneticist working with homozygous or homocaryotic mutant strains is occasionally surprised to find normal phenotypes appearing in his supposedly pure breeding populations. Sometimes these "reversions" to type can be shown to be due to reverse mutation, but frequently they are the result of a mutation at another locus which modifies the mutant phenotype toward wild type. Genes which cause

a wild phenotype despite the presence of a non-allelic mutant gene are called *suppressors*. A suppressor may have no other apparent effect than the suppression of the mutant phenotype in whole or part, but it may, on the other hand, also have a mutant effect of its own. Many examples of suppressors are known, and it will be of value to consider some of them in detail.

Table 37. Some Suppressors Found in *Drosophila melanogaster*

Suppressor Symbol	Character Suppressed	Reference
su²-Hw	Hairy wing (*Hw*) Scute-1 (*sc¹*) Cut-6 (*ct⁶*) Forked (*f*) Bithorax-3 (*bx³*) Bithoraxoid (*bxd*) Cubitus interruptus (*ci²*) Yellow wing color of *y²*	370
su^B-pr	Purple (*pr*)	551
su-s *su²-s* *su³-s* *	Sable (*s*) and vermilion (*v*) Vermilion (*v*) and sable (*s*) Speck (*sp*), sable and vermilion	551
su^S2-v pr	Vermilion and purple (*v* and *pr*)	74
Su-S	Star (*S*)	438
Su-ss	Spineless (*ss*)	439

* Alleles or pseudoalleles; see text. Also called suppressor of vermilion and written *su-v*.

Table 37 lists some of the suppressors found in *Drosophila melanogaster*. It will be noted that a suppressor may be dominant (*Su-S* and *Su-ss*), or recessive as in *su-s*, the suppressor of *sable*, and that it may suppress the mutant phenotype given by a dominant (*Su-S* and *su²-Hw*) or recessive mutant gene. A number of them are obviously not very specific for the character suppressed, particularly *su²-Hw*, *su³-s* and *su^S2-v pr*. Just how specific the others are is difficult to decide, since it is not always clear from the descriptions given in the literature whether they have been tested for interaction with other mutant genes. The otherwise non-specific *su²-Hw* shows a peculiar specificity toward the *scute* series of alleles, for it suppresses only *scute-1* (*sc¹*) and not the others of the series. It shows a similar specificity toward the *cut*, *bithorax*, *cubitus interruptus*, and *yellow* allelic

series. The suppressors of *sable* and *vermilion* have no visible effect alone, and therefore can be recognized only when combined with the mutant genes they suppress. When present alone, the suppressors of *purple* (*su*^{B}-*pr*) and *Hairy wing* give an altered phenotype not related in appearance to the phenotype suppressed, while *Su-S* and *Su-ss*, though giving a wild phenotype when heterozygous, are lethal homozygous.

In *Neurospora* suppressors have been described which relieve the requirement for pyrimidine, proline (425), methionine, inositol (195, 197), acetate (361, 621), pantothenic acid (674), and tryptophan and related compounds (263, 715) in mutants requiring these substances.

The suppressor of certain pyrimidine- and proline-requiring mutants (*su-pyr*) is of particular interest because of its proven interaction with a number of non-allelic mutant genes. Table 38 summarizes the ef-

Table 38. The Effects of *su-pyr* in Combination with Certain Pyrimidine and Proline Mutants

Data of Mitchell and Mitchell (425)

Suppressed Mutants	Characteristics and Requirements of Mutant Alone	Characteristics and Requirements When in Combination with *su-pyr*
37301 (*pyr* 3*a*)	No growth on minimal; requires pyrimidine	Grows on minimal like wild type, but is inhibited by ornithine, proline, citrulline and arginine. These inhibitions completely relieved by lysine.
35401 (*prol* 2) 44207 (*prol* 3)	Grow slowly on minimal; stimulated by proline, ornithine, citrulline, or arginine	Phenotypically identical with wild type
pyr 3*a*, *prol* 2 *pyr* 3*a*, *prol* 3	No growth on minimal; require pyrimidine + proline, ornithine, citrulline, or arginine	Like *pyr* 3*a*, *su-pyr* grows on minimal but inhibited by same compounds; inhibitions relieved by lysine

fects of this gene which alone has no observable effect on the phenotype. When in combination with any one of three non-allelic genes *pyr* 3*a*, *prol* 2 or *prol* 3, it completely relieves the requirements of these mutants, so that the double mutants can grow on minimal medium. The suppression of the proline requirement is complete and the double mutant is otherwise phenotypically identical to wild type. But the double mutant, *pyr* 3*a*, *su-pyr* is not identical with wild type,

for although it grows on minimal in the absence of pyrimidine, it is inhibited by the indicated related compounds. These compounds do not inhibit the growth of wild type significantly. The same inhibition and its relief by lysine is exhibited by the triple mutant *pyr* 3*a*, *prol* 2, *su-pyr*.

This suppressor gene has other peculiarities. It does not suppress pyrimidineless mutants which are non-allelic to *pyr* 3*a*, nor does it suppress 21863, a prolineless mutant which is satisfied by proline only and which is non-allelic to *prol* 2 and 3. Furthermore, when in combination with certain ornithine, citrulline, arginine, and lysine mutants, it modifies the phenotypic expression of these mutants. For example, the double mutant between the suppressor and an ornithineless mutant, 34105 (*orn* 3), (*su-pyr, orn* 3) grows in only trace amounts on minimal medium and is stimulated strongly by citrulline and arginine. The mutant *orn* 3 grows considerably better alone on minimal than when in combination with *su-pyr*, and it is stimulated by ornithine as well as citrulline and arginine. Thus *su-pyr* prevents the utilization of ornithine by a mutant apparently blocked before ornithine. This is, in effect, equivalent to moving a genetic block from one position to another, since the double mutant appears to be blocked between ornithine and citrulline. A similar shifting of an apparent genetic block is caused by *su-pyr* in combination with *lysineless*, 33933 (*lys* 1). *Lys* 1 alone is stimulated by α-amino adipic acid or lysine, but the double mutant *su-pyr, lys* 1 responds only to lysine unless arginine, citrulline, or ornithine is added.

A number of examples of gene interaction in plants involving the inheritance of anthocyan pigments are known which may be interpreted in terms of the suppressor phenomenon. One of the more noteworthy of these concerns the action of the dazzler gene (*Dz*) in *Primula sinensis* (554). *Primula* flowers contain malvidin only in the presence of the gene *K*. When homozygous for the recessive allele *k*, the flowers contain only pelargonidin. If *Dz* is present together with *K*, however, pelargonidin is produced, a small amount in *Dz dz*, *K* plants and a larger amount mixed with malvidin in *Dz Dz*, *K* plants. *Dz* is therefore a partial suppressor of *K*.

The ratios obtained in the F_2 in crosses involving suppressors and the gene suppressed are dependent upon the dominance relationships. Assuming that the suppressor has no observable phenotypic effect alone, or neglecting it if it does, one expects the three different ratios given in Table 36. Some of these ratios show similarities or are identical to the ratios expected in the F_2 with complementary and duplicate

genes. The distinction between complementary genes (9:7 ratio) and duplicate genes, and suppressor activity, is obviously based upon dominance relationships and the definitions of standard or wild type. Whether this indicates any fundamental difference between these various types of gene interaction is a matter for speculation.

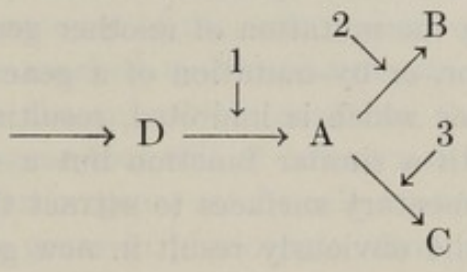
However, it should be noted in connection with the similarity between certain suppressor and duplicate gene ratios that there is adequate evidence to distinguish the two types on the basis of gene action. The suppressor gene might be thought of as a duplicate gene which takes over the role formerly carried out by the normal allele of the suppressed gene. But facts which vitiate against all suppressors being duplicates are as follows: (1) There is no proof that any suppressors are completely specific in their action. Those with proven capacity to suppress the mutant phenotypes of a number of different genes are most certainly not duplicating each of these genes. One could hardly expect that the suppressor of *Hairy wing* duplicates the action of eight other genes or that *su-pyr* replaces *pyr* 3*a* and *prol* 2 and 3. (2) Suppressor genes may produce a mutant phenotype independent of the mutant gene. (3) There are suppressors associated with deficiencies. For example, the *suppressor of Star,* seems to be included within a deficiency (369). One would not expect the absence of a gene to replace the function of another. (4) The combination of the suppressor and the suppressed gene may produce a phenotype which deviates somewhat from wild type. Thus the double mutant strain of *Neurospora,* produced by combining the *suppressor of pyrimidineless* and *pyrimidineless* grows in the absence of pyrimidine, but is extremely susceptible to inhibition by arginine and related compounds. These compounds have no significant inhibitory activity on wild type (303).

Taken together these observations would certainly require that interpretations other than duplication of action are in order with respect to the action of suppressor genes. This is not meant to imply that there are no cases of duplicate action included among those genes called suppressors and vice-versa, but merely that there are two different phenomena, duplication and suppression.

A number of possible mechanisms to explain the action of suppressor genes suggest themselves, and are of general enough interest in connection with gene interaction to warrant some elaboration. Some of these possibilities are as follows: (1) the suppressor gene reestablishes a metabolic balance either by slowing down or speeding up a reaction related to the reaction controlled by the suppressed mutant gene; (2) the suppressor gene provides for an alternative pathway of

metabolism which allows for the formation of a product blocked by the suppressed mutant gene; (3) the suppressor gene reduces the amount or specific activity of an inhibitor which prevents the formation of a required product in the mutant, but not in the wild type.

The applicability of some of these suggested mechanisms can best be appreciated by considering certain simple metabolic systems such as the following one, in which a set of reactions is centered around an



intermediate compound, A, formed in limited amounts from a precursor, D. The production of both B and C is dependent upon A, and it is quite evident that if A is formed in limited amount, and the rate of production of either one is increased the other will be produced in small or negligible amounts. Thus if reaction 2 is speeded up by an increase in enzyme activity, the amount of C formed may be reduced to zero. Hence it would appear as though a block were present at 3. The mutation affecting reaction 2 directly would appear to be more directly affecting reaction 3 since an increase in B may have a negligible effect on the phenotype whereas a reduction in C may have considerable consequences. Suppressor gene action to reestablish a balance, i.e., allow for the required amount of C, could conceivably be through an increase in the activity of an enzyme catalyzing reaction 3, which would then allow for more effective competition for A, or by an increase in activity of the enzyme catalyzing reaction 1, which would provide for more A and hence more C. In either case, the final result would be a reestablishment of the normal phenotype. Any gene mutation resulting in increased activity of enzymes catalyzing either reaction 1 or 3 might therefore be expected to produce a "suppressor" gene.

The possibility that a suppressor gene may provide for an alternative pathway of metabolism should not be overlooked as an explanation for the action of certain suppressor genes. As pointed out by Lein (361) one might expect such an action particularly in the case of those areas of metabolism such as pyruvate oxidation where many alternative pathways are actually known to exist. Lein, therefore, has used

this explanation to interpret the effect of a suppressor which partially relieves the acetate requirement of certain acetate-requiring strains of *Neurospora*. These mutants probably have a dysfunction of pyruvate metabolism, as shown by Strauss (620), which results in the accumulation of acetylmethylcarbinol (acetoin).

It has already been indicated that a genetic block may be the result of an inhibition of the synthesis of some metabolite by another essential metabolite produced in excess (p. 223). Relief of this inhibition may be brought about by the mutation of another gene affecting the synthesis of the inhibitor, or by mutation of a gene controlling the production of the enzyme which is inhibited, resulting in a qualitatively different enzyme with a similar function but a structural configuration with no complementary surfaces to attract the inhibitor. Either type of mutation could obviously result in new genes with suppressor activity.

2. Modifiers in General

To describe a gene as a modifier is to state that it interacts with another gene and modifies the phenotype produced by that gene. The modifier may or may not have an observed effect by itself. All the examples of gene interaction described above involve modifiers which drastically change the phenotype to the wild-type or mutant condition. Many intermediate conditions exist which lie between the extreme examples of suppressors and complementary genes on the one hand, and genes which seemingly produce their phenotypic effect completely independently of all other genes, on the other hand. Most of these intermediate types are best described by the general term modifiers, although here again certain distinctions can be made on the basis of observed effect on the phenotype.

Modifiers may intensify or enhance the phenotypic effect of other genes. Thus the *B* gene in maize is a dominant intensifier of anthocyan pigmentation (159), causing the production of more pigment in those plants which have been determined to have pigment by other anthocyan genes. In *Drosophila* there are intensifiers of certain mutant phenotypes such as, for example, the dominant enhancers of *Minute* (*E-M*(3)*g*) and *Star* (*E-S*) and the recessive enhancer of *Notch* (*e-N*^{8}) (74). A mutual enhancement is found to occur between two recessive mutant genes, *abbreviated* (*abb*) and *shrunk* (*shr*), (74). Flies homozygous for either of these alone show only a relatively weak mutant phenotype and a considerable overlap with wild type. However, the double mutants show complete penetrance for

reduced bristle size and shrunken body. An enhancer may have effects other than causing a more drastic mutant phenotype, as demonstrated by the suppressor of *purple* (su^B-*pr*) which, in addition to its suppressor action, also enhances *Hairy wing*.

Studies on the inheritance of coat color in mammals have shown that in most species there are genes which cause a dilution of the pigmentation of the hair, causing black to become a shade of gray, or brown to become tan, etc. (95, 96, 249). These *dilution genes* are recessive and appear to reduce the amount of pigment present quantitatively without having any other marked visible phenotypic effect. If the main color genes determine the animal to be albino, the effect of substitutions at the diluting gene loci will of course not be recognized. The dominant alleles of dilution genes can be called intensifiers or enhancers.

Related to dilution genes in phenotypic effect, but more drastic in their action, are the *inhibitors*. These are usually dominant genes which prevent the expression of a character such as pigmentation even though all genes involved in the determination of the character are present as the active alleles. They may therefore be called epistatic to the genes determining pigmentation. The dominant genes causing absence of pigment in the chicken (309), the horse (95, 96) and the rabbit, dog and cat (96, 249), are examples of pigment inhibitors. A dominant inhibitor would be expected to give a ratio of 3 standard to 13 mutant in the F_2. The terms inhibitor or epistatic are best not used in connection with recessive mutant alleles. These genes may best be considered as complementary with the gene which they appear to inhibit. An example of this latter type would be the *white eye* gene *w* in *Drosophila* which produces white eyes when homozygous whatever the state of the other eye color genes.

A study of any character in a population comprised of one species, all members of which are classified as the wild phenotype, will always reveal a degree of variability in the expression of the character. Part of the variability can be ascribed to the environment, but genetic tests will usually demonstrate that it is primarily inherent. If the character is one such as length of a part, weight, degree of pigmentation, or the yield of a crop plant which can be determined quantitatively, it will usually be found that the variations fit a binomial distribution. Crossing experiments between variants will give results showing that the character is controlled by a number of genes each of which contributes toward the production of the character, but each having a relatively small effect alone. For example, one can breed for extreme shortness or extreme tallness in an animal or plant by constant inbreeding of

short or tall. This will usually result in shorts and talls which breed fairly close to type, showing that a high degree of homozygosity has been attained. If now the true breeding extremes are crossed, the hybrids will be intermediate, none being either extremely tall or short. Crossing the hybrids will give an F_2 with a wide spread of variation distributed again from the extremely short to the extremely long with most of the offspring intermediate—a pattern of distribution which fits the binomial curve. The genes responsible for this type of continuous character variation are referred to as multiple or quantitative factors. Some authors, such as Mather (406) assume them to be genes which actually do have an intrinsically small effect in the determination of the characteristics of the organism. They are made evident only when they act in an additive or cumulative fashion in groups. Mather calls them *polygenes* to distinguish them from the main genes, or *oligogenes* with large discontinuous effects. When a polygene mutates, or when there is an allelic substitution, the result is the slight type of variation described above. Mutation of an oligogene produces a mutant character which may vary within certain limits, but the variations of the character do not overlap with the character determined by the nonmutant.

The distinction between polygenes and oligogenes is of value in the statistical interpretation of quantitative inheritance. However, there is no proof that a "polygene" is intrinsically any less important than an "oligogene," and the distinction between them is at best highly artificial. It must be recognized that the difference is based upon the degree of mutation and its effect upon the phenotype, and that the extent of observable phenotypic change resulting from mutation of a gene is not a true measure of that gene's significance in producing the total phenotype. Indeed it may be argued that polygenes are of extreme importance in determining the phenotype, and that therefore a drastic mutation of one of them would result in a lethal, or in an oligogene with pronounced effects.

The genes involved in quantitative inheritance must be considered as modifiers of one another, since they act together to produce a particular phenotype. They must also be considered as modifiers of the "main" genes which produce relatively drastic changes in the phenotype, for a mutant character may show the same degree of variability as the wild type or standard. It would follow from this that a main gene produces its phenotypic effect not only by its own activity but subject to the activity of modifiers with assumed slight effects. Proof that this is so is to be found in the results of the vast number of

genetic experiments dealing with quantitative variation of characters. Some of the best examples are to be found in the results of crosses between various strains and species of cotton. Silow (562) has described the effect of crossing the Asiatic cotton, *Gossypium anomalum*, with a leaf type determined by the leaf shape allele L^A to another Asiatic species, *G. arboreum*. By repeated backcross to the *G. arboreum* parent it was possible to transfer the anomalum L^A allele to an essentially *arboreum* background of genes. As will be seen from Fig. 78 the L^A allele produces a quite different phenotype in *anomalum* than it does in *arboreum*. The gene has not mutated, but its phenotypic expression has been altered by changing its environment of modifying genes.

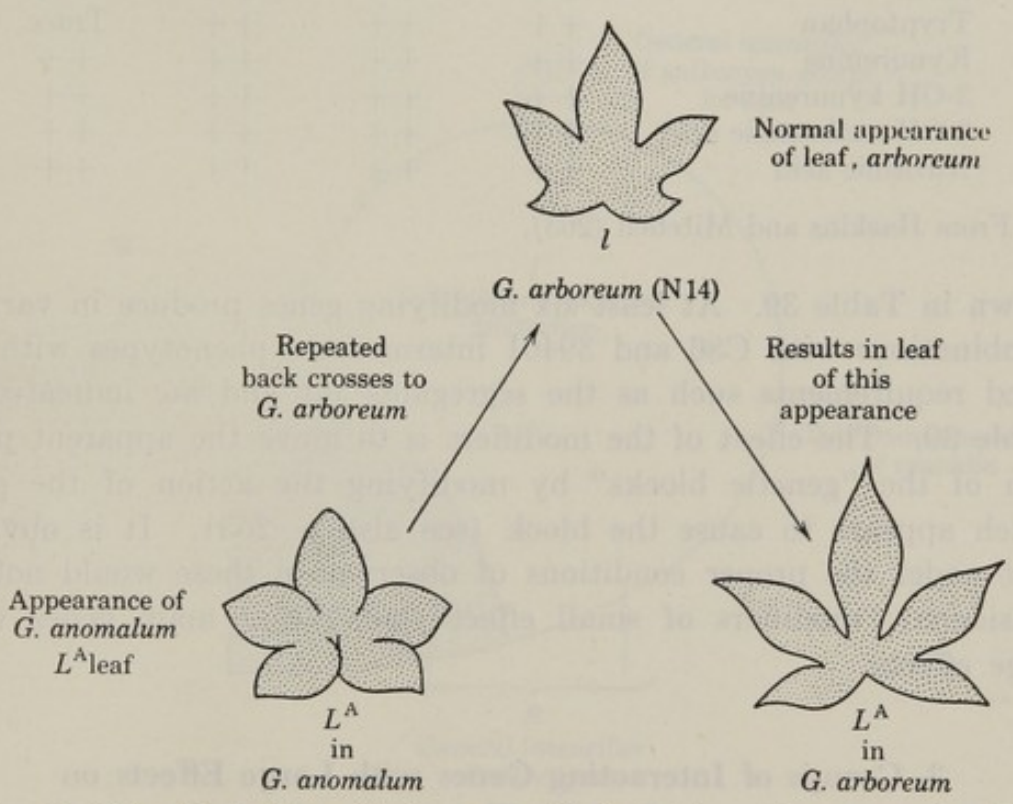


Fig. 78. The effect of transferring gene L^A from *Gossypium anomalum* to an essentially *G. arboreum* background of genes. After Silow (562).

Modifying effects have also been noted on the genes determining nutritional requirements in *Neurospora*, as described for the suppressor of pyrimidine. Another striking example of the modification of a nutritional mutant phenotype is the effect of modifying genes on the expression of the mutant strains C86 and 39401 (263). These are both tryptophanless mutants whose requirements are satisfied by a number of other related compounds in addition to tryptophan as

Table 39. The Effect of Modifying Genes on the Phenotypic Expression of Two Mutants of *Neurospora* Stimulated by Tryptophan *

	C86	*int*	39401	*nic*
Phenylalanine	++	+ to trace	Trace	Trace
Anthranilic acid	++	++	Trace	Trace
Indol	++	++	++	Trace
Tryptophan	++	++	++	Trace
Kynurenine	++	++	++	++
3-OH kynurenine	++	++	++	++
3-OH anthranilic acid	++	++	++	++
Nicotinic acid	++	++	++	++

* From Haskins and Mitchell (263).

shown in Table 39. At least six modifying genes produce in various combinations with C86 and 39401 intermediate phenotypes with altered requirements such as the segregants *int* and *nic* indicated in Table 39. The effect of the modifiers is to move the apparent position of the "genetic blocks" by modifying the action of the gene which appears to cause the block (see also p. 263). It is obvious that under the proper conditions of observation these would not be considered "modifiers of small effect" but rather main genes with large effects.

3. Groups of Interacting Genes with Large Effects on Specific Characters

An insight into the complexities of gene interactions is perhaps best afforded by considering some examples of the effects of groups of genes on a specific character such as pigmentation. The inheritance of flower color pigments has been a favored subject for the investigation of gene interaction. The results have provided adequate evidence for the great complexity of gene interaction, and in addition have brought to light the existence of a number of interesting phenomena which are undoubtedly significant manifestations of genes acting in groups. One of these investigations shall be considered in some detail, and attempts made to analyze some of the results in terms of the simpler examples already cited.

Flower color in the poppy, *Papaver Rhoeas,* is controlled by at least ten known genes (456, 483). Only seven of these are of interest in this discussion, because something is known about their effects on the

distribution and synthesis of the flower color pigments (554). (See Fig. 79.) If the primary effect of each of these seven genes is considered alone, without particular reference to its interactions with the others, it is found that substitutions at each locus of the recessive by the dominant show the following: (1) *C*. The dominant allele is a general intensifier of anthocyan production in the petals, except that

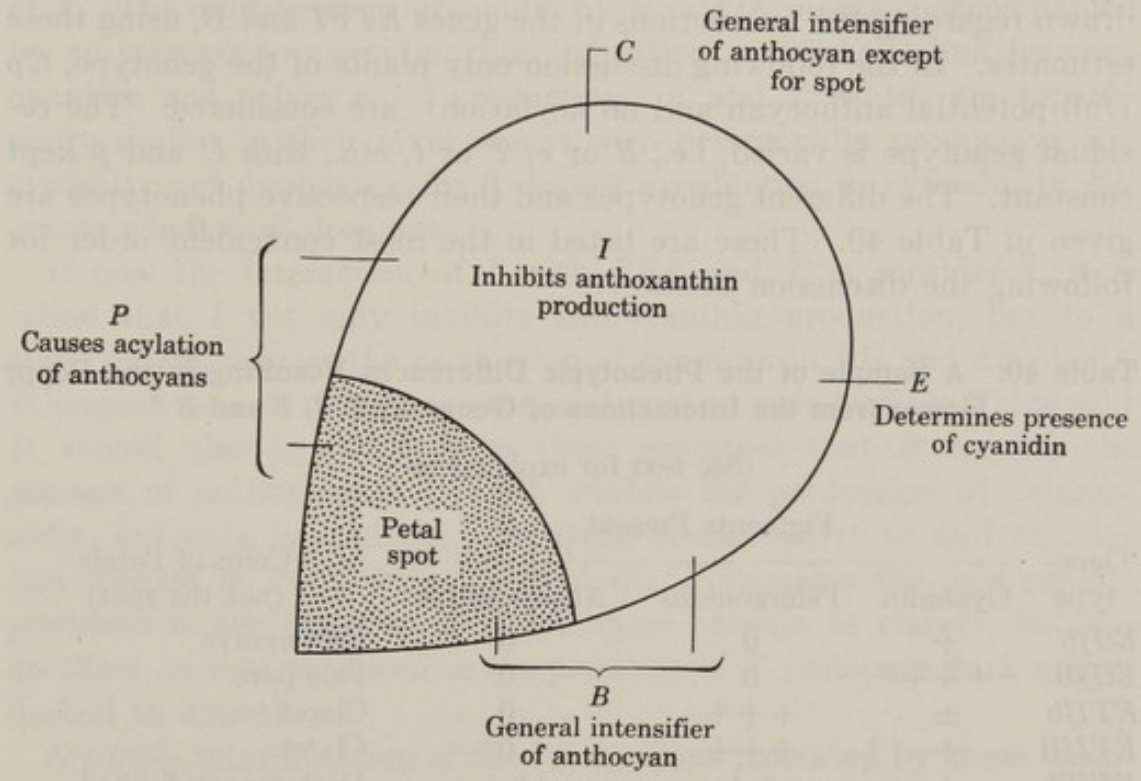


Fig. 79. The effects of genes *C*, *P*, *E*, *B*, and *I* on the color of the poppy (*Papaver Rhoeas*). A single petal is illustrated showing the petal spot.

it has no effect on the pigmentation of the spot at the base of the petal (see Fig. 79). Plants recessive for *c* have very little anthocyan, no matter what the condition of the other color genes. (2) *P*. The anthocyans are acylated in the presence of the dominant allele, and the cell sap acidity of the petals is increased. (3) *E*. The anthocyanidin derivatives are in whole or part cyanidin in *E* plants. Plants homozygous recessive for *e* contain only pelargonidin derivatives. (4) *B*, like *C*, is a general intensifier of anthocyan production, but it also affects pigmentation of the spot. The spot is black in *E* plants and contains cyanidin, and in *e* plants it is brown and contains pelargonidin. Plants of the genotype *bb* always have white spots regardless of the residual genotype. (5) and (6), *T* and *F* are both involved in the production of pelargonidin in *E* plants. No pel-

argonidin is produced in *Etf* plants. (7) *I* is a dominant inhibitor of anthoxanthin production.

Various combinations of the dominant and recessive alleles at these seven loci have been produced and the phenotypes described (456, 483). Over thirty different flower colors have been analyzed by Scott-Moncrieff (554) for their anthocyan and anthoxanthin content and rough quantitative estimates made. Certain conclusions may be drawn regarding the interactions of the genes *ETFI* and *B*, using these estimates. In the following discussion only plants of the genotype, *Cp* (full potential anthocyan and no acylation), are considered. The residual genotype is varied, i.e., *E* or *e*, *T* or *t*, etc., with *C* and *p* kept constant. The different genotypes and their respective phenotypes are given in Table 40. These are listed in the most convenient order for following the discussion below.

Table 40. A Sample of the Phenotypic Differences Resulting in the Poppy Flower from the Interactions of Genes *E, T, I, F* and *B* *

(See text for explanation.)

Geno-	Pigments Present			Color of Petals
type	Cyanidin	Pelargonidin	Anthoxanthin	(not the spot)
EtIfb	+	0	0	Pale mauve
EtIfB	+++	0	0	Pale port
ETIfb	±	+++	0	Claret
ETIfB	+	+++	0	Claret
ETiFb	+	++	++	Dark mauve flushed
EtiFB	+++	+	+	Brownish port
etifb	0	+	++	Lilac
etifB	0	+	++	Lilac
etiFb	0	+++	++	Dull carmine lake flushed
etiFB	0	+++	++	Dull carmine lake flushed
Etifb	++	0	++	Mauve
EtifB	++++	0	+	Port
etIFb	0	++	0	Almost claret
ETifb	+	++	++	Petunia

* From Scott-Moncrieff (554).

The relationships of *E*, *B*, *F*, and *T* are best understood by referring to changes at the *B* locus. All five of these genes affect anthocyan production as noted previously. *B* increases the production of cyanidin in *Et* plants when it replaces *b*, but has little or no effect on the production of pelargonidin or cyanidin produced in the presence of *T* in *ET* plants. On the other hand, *B* reduces the amount

of pelargonidin in *EtF* plants; and has no effect on the production of pelargonidin in *eF* or *ef* plants. There seems to be no effect of *B* on the presence of pelargonidin in *ETF* plants.

Recalling the effect of *B* and *b* on the petal spot, it would seem from these observations that *B* has only a very indirect effect on pelargonidin production in the petals in parts other than the spot. Furthermore, its intensification of cyanidin occurs only in the absence of *T*. The dilution of pelargonidin by *B* in *EtF* plants is accompanied by an increase in cyanidin, thus indicating a metabolic link between cyanidin and pelargonidin production. *F* and *T*, which are superficially similar in their involvement with pelargonidin production, are evidently not duplicates, for *T* causes a reduction in cyanidin in *EB* plants whereas *F* does not.

If now the interaction of *I* with *EBF* and *T* is considered, it is found that *I* not only inhibits anthoxanthin production, but to a slight extent reduces the production of cyanidin in *EB* and *Eb* plants. (Compare mauve to pale mauve, and port to pale port, in Table 40.) It should also be noted from these examples that *B* reduces the amount of anthoxanthin. *I* also inhibits the production of pelargonidin, but only in *e* plants; (compare almost claret to dull carmine lake flushed in Table 40). In *E* plants, *I* increases the pelargonidin produced in the presence of *T* (compare petunia to claret), but has no effect on that produced in the presence of *F* (compare dark mauve flushed to claret).

A simple interpretation of the interactions indicated by these examples in the poppy is not possible using the terms given in the preceding sections. The gene *B* may be considered as an intensifier of *E*, since it increases the production of cyanidin in the presence of *E*, but it is also a dilution factor, since it reduces anthoxanthin in *i* plants and pelargonidin in *EF* plants. The gene *I* is an inhibitor of anthoxanthin, dilutor of cyanidin and pelargonidin, and under certain conditions an intensifier of pelargonidin. *T*, aside from its role as a specific producer of pelargonidin in *E* plants, is a suppressor of *B*, for *ETIfb* and *ETIfB* give an identical phenotype (claret). It is only by the presence of the spot in *B* plants that the gene *B* is recognized when *T* is present. These considerations show clearly that each one of the five genes fits into more than one of the categories of gene interaction described in the previous sections. It is quite evident that conclusions regarding the action of a gene must take into account first the aspect of the phenotype studied and secondly the condition of the other genes which also have effects on the same

phenotype. If a gene's action, at the level at which we are forced to study it in the case of plant pigments, is the result of interaction with other genes, it follows that the observed effect on the phenotype may be very indirectly related to the gene's primary effect.

As complicated as the picture may become as a result of an analysis of the pigments of the poppy flower, it is nonetheless evident that the observed interactions may in part be the result of competition for

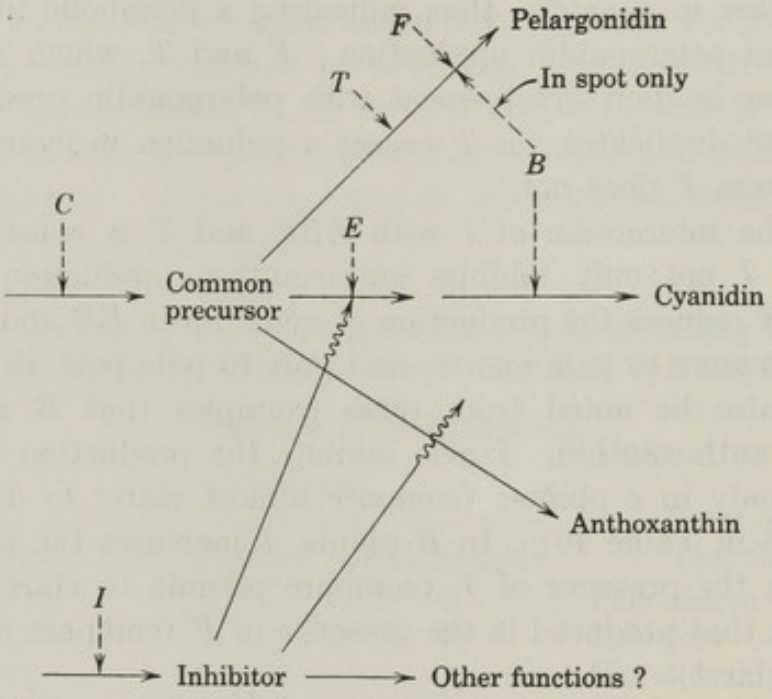


Fig. 80. An interpretation of the actions of the poppy flower color genes based on the assumption of competition for a common precursor.

common precursors used in the production of the final pigments. A common precursor substrate is indicated for the anthocyans and the anthoxanthins similar to the example given for *Dahlia* on p. 82. *I*, in causing an inhibition of anthoxanthin, also causes a reduction of anthocyans in certain genotypes. Competition between cyanidin and pelargonidin production is apparent from the interactions of *T* and *B*, and *F* and *B* in *E* plants. A tentative explanation of the interactions based on the assumption of competition for a limited common precursor is given in Fig. 80. It is necessary, for this interpretation to apply, to assume that the recessive alleles, *b*, *t*, and *f*, are hypomorphs in their control of the pigment production in parts other than the spot. The gene *I* may be very indirectly related to the production of these pigments through its control of some hypothetical inhibiting compound which affects anthoxanthin and cyanidin produc-

tion, the first to a greater extent than the second. Since this inhibition would be expected to relieve demands upon precursor material, pelargonidin would be expected, as it does, to increase.

Differences in Melanin Pigment in the Guinea Pig

One of the most elegant and thorough investigations of pigment inheritance to be found in the literature is that conducted by Wright and his associates over a period of more than 30 years on the melanin pigments of the guinea pig. The results of these investigations have demonstrated that the production of melanin in animals is the resultant of the interaction of a large number of genes.

Melanin is the primary pigment found in the vertebrate integument and the integumentary derivatives such as hair, feathers, and scales. Its chemistry is not understood, although it is certain that oxidized substances derived from tyrosine are involved in its formation. Two types of melanin are generally defined on the basis of color: the dark melanins or eumelanins and the yellow-orange-red melanins, or phaeomelanins. Melanin is deposited as microscopically visible granules within specialized cells, the melanophores localized in the integument. It is introduced into the integumentary derivatives during their formation.

At least nine gene loci are known in the guinea pig which have effects on the color of the hairs. Four of these are primarily pattern genes whose effects will be disregarded in this discussion which will be restricted to the inheritance of pigmentation in uniformly (self) colored animals. (See Table 41.)

The gene *E* determines the presence of eumelanin; its recessive allele, *e*, gives a phaeomelanic animal. Two qualitatively different eumelanins are apparently produced in eumelanic animals, brown in *bb* animals and sepia in *B* animals. Both sepia and brown pigment may be reduced in intensity in the presence of the recessive allele of the gene *P*. The yellow, phaeomelanic pigment is affected quantitatively only by the gene *F* which is incompletely dominant. *FF* animals may be intense yellow, with *Ff* and *ff* genotypes having correspondingly less yellow pigment in that order. *F* has no effect on eumelanic animals in the presence of *P*, but *ffpE* animals have no eumelanin and may have a small amount of phaeomelanin.

The intensity of the pigmentation produced by *E*, *P*, *F*, and *B* and their respective alleles is determined by a series of five *albino* alleles, C, c^k, c^d, c^r, and c^a. C is in most cases completely dominant over

Table 41. Quantitative Estimates of the Amount of Melanin in the Hair of Guinea Pigs *

c Alleles	Eumelanic				Phaeomelanic				Eye Color		
	Sepia		Brown		Yellow and Red				*Ec*; *Ff* without Effect		
	EPFB *EPfB*	*EpFFB*	*EPFb* *EPfb*	*EpFFb*	*EpfB* *Epfb*	*cpFFB* *ePFFB* *cpFFb* *ePFFb*	*cpFfB*	*cpffB*	*PB*	*Pb*	*pB* *pb*
C-	100	17	100	28	6.2	100	85	34	Black	Brown	Pink
$c^k c^k$	89	15	96	23	0	36	31	4.5	Black	Brown	Pink
$c^k c^d$	82	11	102	17	0	39	30	5.9	Black	Brown	Pink
$c^d c^d$	67	5.3	85	14	0	36	33	5.1	Black	Brown	Pink
$c^k c^r$	94	9.1	97	16	0	18	13	—	Black	Brown	Pink
$c^k c^a$	75	6.2	83	13	0	17	14	0.5	Black	Brown	Pink
$c^d c^r$	72	3.3	96	7.3	0	14	12	0.3	Black	Brown	Pink
$c^d c^a$	39	2.4	63	4.0	0	14	14	0.5	Black	Brown	Pink
$c^r c^r$	81	2.0	95	4.1	0	0	0	0	Dark red	Dk. brown-red	Pink
$c^r c^a$	42	0.5	64	0.9	0	0	0	0	Light red	Lt. brown-red	Pink
$c^a c^a$	0	0	0	0	0	0	0	0	Pink	Pink	Pink

* From Wright (712).

the lower alleles and gives the most intense pigmentation. Animals homozygous for c^a are albinos with pink eyes. The other alleles give intermediate intensities.

Despite the lack of knowledge of melanin chemistry, it has been found possible to make quantitative estimates of the amount of melanin in the hairs of various colors and shades of guinea pigs by gravimetric and colorimetric methods after extraction of the melanin with suitable solvents (269, 524, 173). In addition Wright (712) has estimated the relative amounts of pigment in the different phenotypes by using a series of standard skins. The estimates obtained by the colorimetric method agree quite closely with those determined by means of the empirical grades (712, 713) so that a fair degree of confidence may be placed in the estimates. Table 41 gives the most recent data obtained by using the empirical grades as reported by Wright (712), and includes descriptions of the eye colors produced by the various genotypes. In each case the estimates for the hair melanin are given on the basis of the most intense sepias, browns

and yellows being taken as 100%. Some of the data are plotted in Fig. 81 for convenience in analysis.

The *C* alleles express themselves differently in the eumelanic and phaeomelanic animals. In the browns and sepias there are five alleles, which are listed here in increasing order of effect, c^a, c^r, c^d, c^k, and *C*. The phaeomelanic, *ee* animals, however, can give evidence for the existence of only three alleles designated as c^{ra}, c^{kd}, and *C*; for $c^a = c^r$, and $c^k = c^d$. Within the eumelanic animals, the dark-eyed sepias and browns (*EPB* and *EPb*) show peculiar effects with the different combinations of the *C* alleles. There are a series of "waves" of effect when the data are plotted as they are in Fig. 81. These undulations are not evident in the pink-eyed sepias and browns. If the quantitative effect of the *C* alleles on sepia in *EPB* animals is compared to their effect on the yellow pigment in *eFF* and *eFf* animals, a correspondence will be noted between the dilution in the sepias in the presence of c^dc^a and c^dc^d, and the intensification of the yellow pigment. On the other hand, the combinations: c^dc^a, c^dc^r, c^kc^a, c^kc^r, and c^dc^d, c^kc^d, c^kc^k give quantitatively the same amount of phaeomelanic pigment, but there is an increase in eumelanin in their presence. Wright (709, 710) has given a detailed interpretation to explain this phenomenon in terms of competition of the phaeomelanin and eumelanin synthetic mechanisms for common substrates and enzymes.

The genes *P* and *b* both reduce the concentration of eumelanin. The reduction by *b* is not evident in Table 41, since the most intense *brown* has been assigned a value of 100; actually there seems to be a decrease of about ⅓ that present in the sepia animals. In addition to a quantitative reduction, *b* also produces a qualitatively different pigment, brown instead of sepia. There seems to be no effect of *p* on eumelanin other than that it reduces both brown and sepia and eliminates eumelanin completely in the presence of *ff*.

The activity of an enzyme presumed to be of importance in the production of melanin in the guinea pig, has been investigated by W. L. Russell (529) and Ginsburg (199) in an attempt to correlate enzyme activity with observed phenotypic effects. The problem was approached by assuming that dopa (dihydroxyphenylalanine), an oxidation product of tyrosine (hydroxyphenylalanine), is an intermediate in the production of melanin. This is an assumption which has considerable experimental evidence in its favor, but there is no direct evidence for it in the guinea pig, other than that in the presence of dopa a black pigment is formed by those tissues, and their extracts,

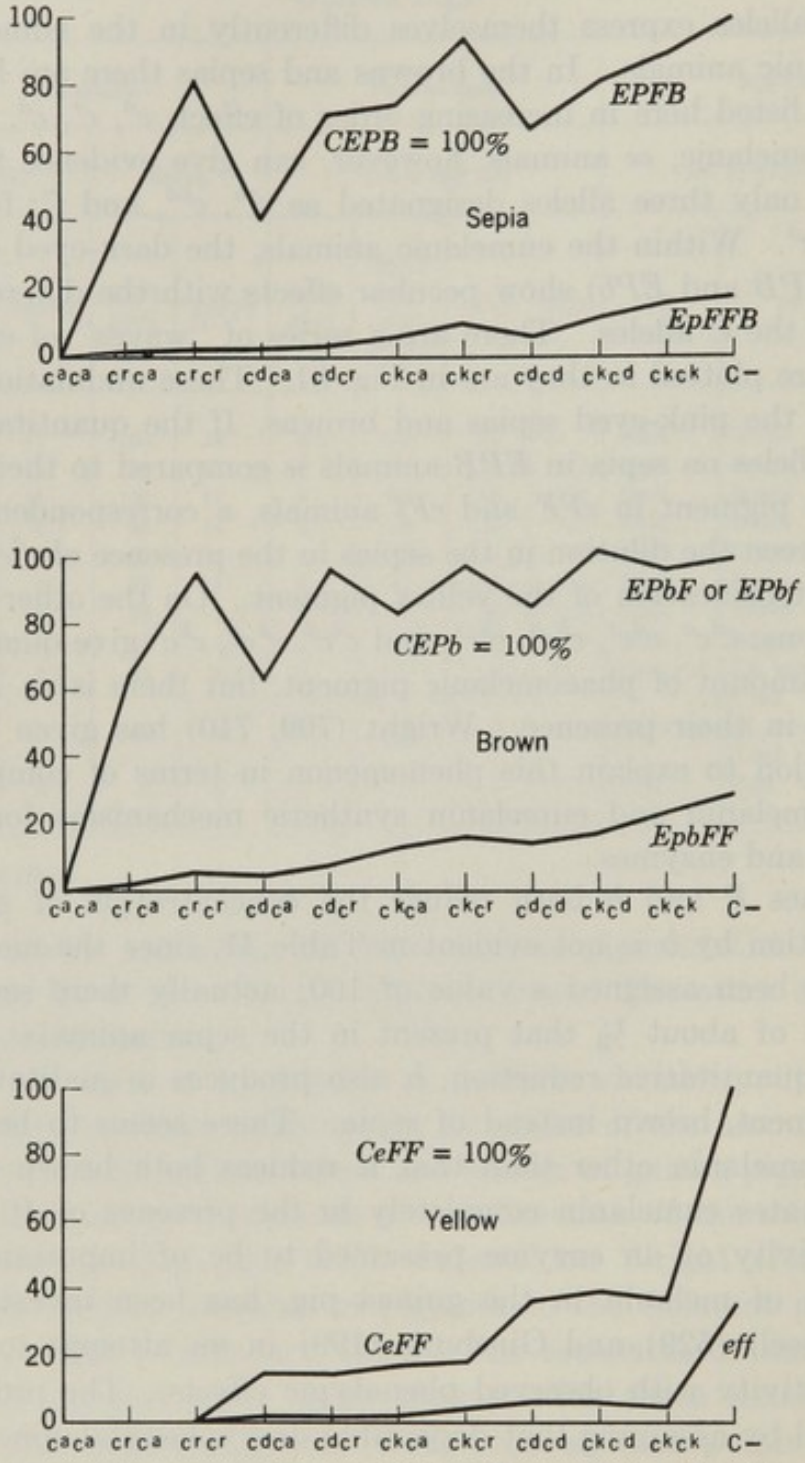


Fig. 81. A graphic representation of the effects of the *C* series of alleles in different genetic backgrounds in the guinea pig. After data of Wright (712).

which are most active in producing natural melanin. The pigment formed from externally introduced dopa is black even in what seem to be pure phaeomelanic guinea pigs. The results of testing extracts and tissue slices of guinea pig skin from a large number of different genotypes for dopa oxidizing activity (dopa oxidase) were quite clear in indicating that substitutions at the *E*, *P*, and *B* loci have little or no effect, and that *C* and *F* have definite effects. The activity of the *C* alleles in *both* the eumelanic and phaeomelanic animals is restricted to four levels corresponding to the effects of three alleles *C*, c^{kd}, and c^{ra}. These correspond to the same three alleles which are expressed in phaeomelanic *ee* animals. Substitution of *f* for *F* has a similar reducing effect on oxidase activity in both pigment types. The conclusion has been drawn therefore that the enzyme measured by its activity for dopa oxidation is concerned primarily with phaeomelanin production (199, 529). The dopa oxidase activity of the active melanin-producing tissues of the house mouse have also been tested, with essentially the same results. A correspondence of dopa oxidase activity with the levels of phaeomelanic concentration is indicated, but little or no correspondence with changes in eumelanic pigment (528) were noted.

The nature of pigmentation in the house mouse has been investigated in great detail by E. S. Russell (525, 526, 527) with respect to four characteristics: (*a*) nature of granule color, (*b*) degree of pigmentation, (*c*) size of granules, (*d*) clumping of granules. All four of these are of significance in determining the final coat color. The effects of gene substitution on these different aspects of pigmentation are quite striking, and can be effectively studied by observing the condition of the hair follicles in section.

In general it was found that the *C* series seems to affect only the degree of pigmentation by changing the number of granules or reducing their size, and that the *P* locus (corresponding in effect to *P* in the guinea pig) produced a similar reduction in volume, but by means of changing the shape of the granules to shreds as well as reducing their size. Qualitative pigment changes in the granules were obvious in substitutions at the *B* (brown) and the A^y loci. Both of these seem to control the type of pigment present, black or fuscous to brown in the *B* to *b* substitutions, and eumelanins to phaeomelanin in the A^y to *a* changes. The change produced by *B* in the quality of the granular pigment is accompanied by a change in shape and total volume of the pigment. The so-called dilution gene, *d* in the mouse, which has no counterpart in the guinea pig, appears from vis-

ual impressions of the coat color to reduce the total volume of pigment in both eumelanic and phaeomelanic animals when it replaces dominant *D* which gives no dilution. However, histological examination reveals that substitution of *D* by *d* does not reduce the volume of pigment (actually it appears to increase it) but that the apparent dilution is caused by a clumping of granules.

These observations are of considerable significance in the analysis of coat color differences. They make it clear that a chemical approach to the problem, even provided the chemistry of the melanins were understood, must be accompanied by morphological and developmental considerations.

As in the case of plant pigments, investigations into the inheritance of melanin pigments in vertebrates typified by the examples given above reveal that a simple character such as pigmentation has a complicated genesis which involves the interactions of numerous genes. Only a few of these genes are generally recognized by the geneticist, for he is limited to the viable mutations affecting coat color. This we must consider to be true for all aspects of the phenotype, not only pigmentation, even though the best evidence for the complexity of interactions is found in the inheritance of pigmentation. Undoubtedly the reason why interactions are so easily studied in connection with pigmentation is that the genes affecting it may mutate to recognizably different alleles without grossly affecting the well-being of the organism, as would be the case if genes directly controlling important physiological functions were to mutate to amorphic or weakly hypomorphic states and cause lethality. One of the chief reasons why the work with *Neurospora* is so significant is that it enables the geneticist to overcome this situation by using those mutants which, although lethal on minimal medium, respond to diffusible growth substances. It is to be expected that studies on gene interaction in this organism, and others like it, will vastly extend our knowledge of this subject beyond the point reached by the pigmentation investigations.

4. Genic Balance

The organism is the resultant of thousands of genes acting in concert. Its phenotype results from the blended, balanced, and synchronized activities of the products of all genes in the genome. On this point of view all genes interact with one another through their products, and are therefore modifiers—modifiers of the activities of one another. In order for the modifying system to act harmoniously to

produce a functional organism the system must be in balance—this is *genic balance*. It is necessary, in other words, not only to have a full complement of genes, but a complement of genes present in the proper dosage relations necessary to produce a balanced system.

In a sense the concept of genic balance receives its support from all experiments which show the dependence of a specific character upon more than a single gene, but the idea is perhaps best brought out by considering the normal process of determination of sex in *Drosophila*. In this species, as in nearly all organisms with sexual dimorphism, sex is determined by the chromosomes. There is a mechanism which acts to produce an approximately equal distribution of sexes, and in *Drosophila* it appears to be associated with a balance between the genes on the autosomes versus those on the X-chromosome. Bridges (73) has described the effects of changing the normal chromosome complement of *Drosophila melanogaster* (2X's and 2 pairs of each autosomal type in the female, and XY with 2 pairs of each autosomal type in the male) on sex. Some of the combinations and the results are given in Table 42. In this table, 1A

Table 42. The Effect of Changing the Ratio of X-chromosomes to Autosomes on the Determination of Sex in *Drosophila melanogaster*

Super Female	3X:2A
Female	4X:4A
Female	3X:3A
Female	2X:2A
Intersex	3X:4A
Intersex	2X:3A
Male	1X:2A
Male	2X:4A
Super male	1X:3A

refers to each of the autosomes as being present once, 2A each twice, etc. It will be noticed that females are produced whenever the ratio of X-chromosomes to autosomes in each of the homologous groups is 1:1. Exceptional females, the so-called "super females" result from a ratio of 3X:2A. If now the number of X-chromosomes is reduced relative to the number in each group of autosomes, there results normal males (1X:2A:2X:4A) and "super males" (1X:3A). Individuals with both male and female secondary sex characteristics, the so-called "intersexes," arise as a result of ratios intermediate between

1X:2A and 1X:1A. Thus 3X:4A and 2X:3A individuals are intersexes. The Y-chromosome normally present in the male apparently has no role to play in sex determination for 2XY:2A females are typical females and XO:2A individuals are definitely male in appearance, although sterile.

The nature of the ratios, in which a female is always produced when the X-chromosomes are equal to or greater in number than the autosomes in the homologous groups, would indicate a preponderance of female-determining capacity in the X. This could be due to a single gene or a group of genes on the X. Extensive tests by means of special techniques involving duplication of small segments of the X in males and intersexes have proved that there is no one gene on the X or even a group of genes close together which will cause a male or intersex to become female (120, 144, 476, 485). The multiple female-determining genes are scattered almost at random, and they express themselves cumulatively in the female direction in conjunction with genes on the autosomes which may be considered to be acting in the direction of maleness.

Even though under normal conditions the sex of *Drosophila* can be changed only by manipulating blocks of genes on the autosomes and X-chromosome to establish a new balance, this does not mean that sex cannot be changed by mutation of a single gene. A number of autosomal gene mutations have been described which convert diploid (2X:2A) females into sterile males or intersexes in *D. melanogaster* (219, 626) and in *D. virilis* (355, 613). Thus, despite the fact that a large number of genes are known to be involved in the determination of sex, a single gene change may cause a complete or partial reversal of sex. This may seem to be a paradox, but in reality it is precisely what one would expect in view of the preceding discussion in this chapter. Among the many genes acting together to produce sex organs and other secondary sexual characteristics, there are those which must be present in certain relative frequencies in order for one or the other sex to be produced. These latter may be acting against one another, one set in the male direction, the other in the female direction, as the ratios of X-chromosomes to autosomes would indicate. It is therefore likely that a mutation of any one of them by reducing or increasing the dosage activity sufficiently toward producing one sex, would cause a reversal of sex, even though all other genes are unaffected.

Support for the concept of genic balance is by no means confined to the results of analysis of sex determination in *Drosophila*. In-

vestigations of sex determination of different animal species such as the gypsy moth, *Lymantria* (206), the guppy fish, *Lebistes* (698), the wasp, *Habrobracon* (686), and others, have resulted in data consonant with the idea of genic balance.

By utilizing the technique of duplicating or reducing various sections of the genome, it is possible to show that the genic balance mechanism acts for characters other than sex. In some plant species individuals frequently arise with one or more extra chromosome in addition to the normal complement, a condition related to the aberrant chromosomal complements discussed in the preceding section in connection with sex determination. The general term applied to this condition is *aneuploidy*. Figure 82 illustrates some aneuploid types.

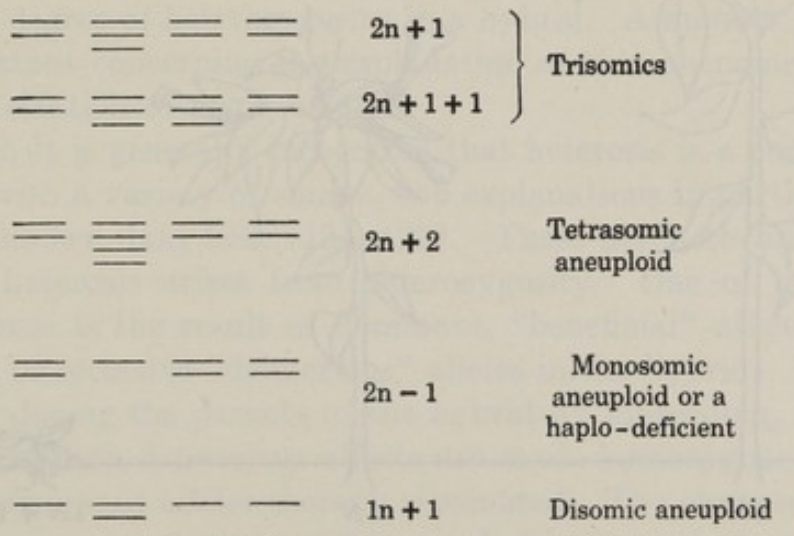


Fig. 82. Some types of aneuploids.

Trisomic aneuploids ($2n + 1$, $2n + 1 + 1$, etc.) are usually readily distinguishable by phenotype from the normal diploid or the almost normal triploids or tetraploids. The addition of extra chromosomes in both the Jimson weed, *Datura* (47), and tobacco, *Nicotiana sylvestris* (215), has a distinct effect on the phenotype. The phenotypic alterations produced depend upon the chromosome duplicated and the size of the duplicated piece, if it be only a portion of a chromosome. They may vary in type from changes in over-all size of the plant, shape of leaves and flowers, and in a number of physiological characters such as time of flowering, etc. Extreme divergence is noted particularly in haploids of *Datura* which are disomic. As shown in Fig. 83 the haploid is smaller than the normal diploid, and the addition of a single chromosome does not carry it toward the diploid phenotype, but to an extreme degree in the opposite direction.

Abnormal development reflected primarily in changes in fertility and viability is also obtained in *D. melanogaster* when sections of chromosomes are duplicated (81, 475). This is true for duplication of parts of both the sex chromosomes and autosomes, although changes are more drastic when certain parts of the autosomes are duplicated than when the sex chromosomes are duplicated. This indicates an intrin-

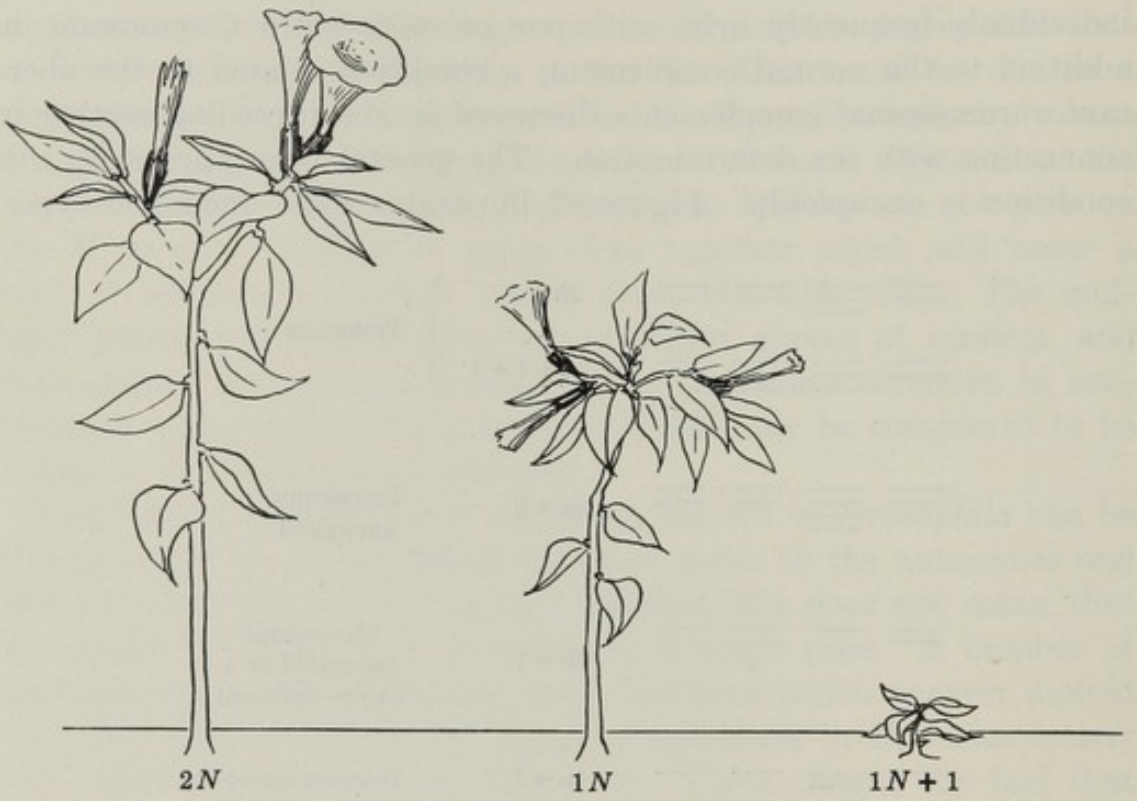


Fig. 83. A comparison of diploid, haploid, and disomic *Datura* plants. Copied from a photograph published in the *Journal of Heredity* after Satina, Blakeslee, and Avery (537).

sic tolerance toward duplication on the part of the X associated with its role in sex determination, a fact no doubt related to its being regularly haploid in males and diploid in females.

Crosses between different species frequently result in the appearance of sterile progeny, or progeny which are strikingly different from either of the parent forms. Sterility in species hybrids has been especially studied in *Drosophila* species. (See Patterson and Stone (474).) Gross changes in morphology in *Drosophila* species hybrids have been reported in crosses between *D. athabasca* and *D. azteca*. The hybrids in this case may be giants or dwarfs compared to the parents, depending on the type of cross made (628). Similar morphological changes also result from plant interspecific crosses such as

in mosses (685*a*) and flowering plants (569). Some of these results are to be attributed to non-disjunction resulting in aneuploids, but for the most part the abnormal hybrids are regular diploids or polyploids and not comparable to the aneuploids discussed above. Nonetheless the general explanation is the same; the combining of two somewhat dissimilar complements of genes into one individual results in an unbalanced genetic system which cannot function in a normal fashion.

On the other hand, hybrids resulting from crosses between unrelated strains frequently show a marked enhancement of a particular character or group of characters which are of adaptive value to the organism or of economic value to the breeder. This phenomenon is termed *heterosis* or *hybrid vigor,* and it is the direct result of achieving a high degree of heterozygosity in a hybrid. A number of hypotheses are extant concerning the explanation of this phenomenon which is a manifestation of genic balance.

Although it is generally recognized that heterosis is a complex phenomenon with a variety of causes, two explanations in particular seem to fit the known data best (121, 122). These are both based on the fact that heterosis arises from heterozygosity. One of them states that heterosis is the result of dominant, "beneficial" alleles masking the effects of recessive "deleterious" alleles in the hybrid. It assumes that in producing the parents of the hybrid by inbreeding, certain recessive genes with deleterious effects are made homozygous, and their beneficial dominant alleles thereby eliminated. The chances are slight that the same sets of genes will be made homozygous for deleterious recessives in any two independent strains by inbreeding, and hence when these are crossed many of the recessives will be covered by dominants in the hybrid.

A second hypothesis is based on the assumption that heterozygotes resulting from two alleles, for example a_1 and a_2 in combination may be more "vigorous" than the respective homozygotes, a_1/a_1 and a_2/a_2. This is actually more than an assumption, for it has already been noted that in certain allelic series antimorphs will produce, in heterozygotes, phenotypes which are unexpected on the basis of their action when homozygous. The action of pseudoalleles in heterozygotes will also be recalled. Furthermore, many extreme cases of complementary action of what appear superficially to be allelic genes have been recognized in which genes giving a distinct mutant phenotype when homozygous produce a normal phenotype characteristic of the normal dominant allele when heterozygous. The maize mutant gene

yg_2 when homozygous results in seedlings with yellow-green color, but in combination with a mutant allele, *pale yellow,* (*py*), the heterozygote is normal green. The explanation for this unexpected interaction between yg^2 and *py* seems to be that *py* is associated with a deficiency (Fig. 66, p. 227) which does not include the yg^2 locus. Since the *py*-bearing chromosome carries the normal allele, Yg^2 and the yg_2 chromosome is not deficient, the two complement one another and the phenotype of the heterozygote is normal.

Other hypotheses may, of course, be formulated to explain heterosis in its varied aspects. From what is known about gene interaction the possible hypotheses to explain specific cases are almost limitless in number. But the basic principle of all of these explanations will of necessity be that different gene combinations from the same restricted pool of genes will give many different phenotypes—many more than would be expected by considering the action of each gene independently without reference to the results of its interactions.

Although experimental data of a biochemical nature necessary to establish a biochemical basis for phenomena such as heterosis are lacking at present for diploid plants and animals, certain experimental results from *Neurospora* may be useful in the future for aiding in the explanation of these phenomena in the higher forms (166, 163, 510). For example, the possibility of inhibition being the cause of certain partial or complete genetic blocks has already been illustrated with *Neurospora* (p. 223). It is apparent from this that the relief of such an inhibition by reduction of the inhibitory agent in heterozygotes may well be one biochemical explanation for heterosis. Emerson (166) has presented biochemical models based on known situations in *Neurospora* mutants which serve to further illustrate some of the biochemical possibilities.

5. Modifiers of Allelic Expression and Interaction

The relations between alleles, particularly as regards dominance, can be modified by alterations in the genetic background. The genes controlling pigmentation in the *Dahlia* flower are of interest in this connection. When, for example, the mutual modifying activity of *A* and *I* in producing anthocyan and apeginin was considered, it was seen that *A* causes the production of anthocyan at much lower dosages when *I* is absent. Thus *I* modifies the dominance of *A* over *a*. *A* in turn modifies *I* in the same way with respect to apeginin production.

Dominance modifiers have also been demonstrated in diploids, even though the dosage modifications so easily obtained in polyploids such as *Dahlia* are not possible. The vestigial gene, *vg* in *Drosophila,* is an excellent example. The mutant alleles at the *vg* locus cause modifications of the wing size and shape. It has been shown that the dominance of vg^+ over *vg* and other mutant alleles (vg^{no2}, vg^{nw}, etc.) can be greatly reduced by (1) an allele at the cut locus, $ci^{do\text{-}vg}$ (207) and (2) by a number of different *Minutes,* $M(2)l^2$, $M(1)n$ and $M(3)w$ (226). Some data showing the effects of the minutes are given in Table 43. The vestigial alleles like the *ci* alleles have incomplete

Table 43. The Effect of Certain Minutes on the Dominance of vg^+ over Its Recessive Mutant Alleles *

$vg/+$	Per Cent Normal Flies	$vg^{nw}/+$	Per Cent Normal Flies	$vg^{no2}/+$	Per Cent Normal Flies
Alone	99.2	Alone	97.78	Alone	95.3
$M(2)l^2$	68.8	$M(2)l^2$	12.10	$M(2)l^2$	5.4
$M(1)n$	27.6	—	—	$M(1)n$	—
$M(3)w$	12.4	$M(3)w$	0.00	$M(3)w$	0.0

* From Green (226).

penetrance. Dominance modification by the *Minutes* and $ci^{do\text{-}vg}$ consists in increasing the penetrance in heterozygotes with + and also in causing an increase in the expression of the intensity of the mutant phenotype. These modifiers may therefore be considered as dominance modifiers of the + alleles as well as enhancers of the mutant alleles. The flies which are homozygous for $ci^{do\text{-}vg}$ are wild type unless in combination with mutant *vg* alleles. *Minute* flies have reduced bristles and a longer life cycle primarily due to prolongation of the larval period.

As a result of evolutionary considerations Fisher (180) has concluded that the degree of dominance of a gene is conditioned by modifiers selected to make the heterozygotes with other alleles equivalent in phenotype to that produced by the gene homozygous. This assumes, of course, that the phenotype determined by the dominant is the best adapted to the environment—a valid assumption provided one accepts the theory of natural selection. Therefore it is expected that the wild-type allele will be one of the dominants in any allelic series. This is true for most cases except for domesticated animals and plants in which many of the "wild-type" alleles have become un-

recognizable as such owing to artificial selection of more desirable characters, which also show dominance in inheritance.

There is no doubt that modifiers of dominance exist and that they probably play some role in the evolution of dominance, but it must also be recognized that the difference in potency of alleles, considered as an intrinsic capacity to produce more or less primary gene product, is another factor to be considered in any explanation of dominance. This is after all a restatement of the fact that the phenotypic expression of any gene is determined by its primary action and the result of its interaction with other genes. Which of these factors is more important in the evolution of dominance of the wild-type allele, selection for modifiers or selection for the most potent alleles, is a question which lies beyond the scope of this discussion. The reader is referred to the works of Fisher (180), Wright (708, 710), Haldane (250), and Muller (449) for a detailed discussion of this important problem.

Allelic expression is affected in other ways by modifying genes besides the modification of dominance. The number of alleles in an allelic series which may be recognized is subject to the condition of other genes. This has been illustrated in the previous discussion of the guinea pig *C* series of alleles in which it was shown that animals with pheaomelanic pigment (*eF*) are affected differently by substitutions of those alleles than eumelanic (*EF* or *Ef*) animals. In the phaeomelanic animals only three *C* alleles are recognizable whereas five are necessary to explain the variations in eumelanic pigment. It will also be recognized that suppressor genes prevent the recognition of allelic changes of the genes they interact with when they are present in such a state as to cause the suppression phenomenon. On the other hand, the gene suppressed when in the normal state will mask certain allelic changes of the suppressor gene. From these and other examples it is abundantly clear that not only the number of recognizable alleles of a gene is modified by the condition of other genes, but the measurement of the absolute mutation rate of a gene to other allelic states is considerably handicapped by the possibility of many of the alleles not being capable of expressing a different phenotype from the gene from which they were derived.

CHAPTER 11

Environmental Modification of Phenotype

Phenotypic changes incident to mutation have been the major concern of the preceding chapters. Genetic control of the phenotype is, however, only one aspect of the production of the phenotype. Just as gene changes modify the internal environment of the cell and hence the phenotype, so does the external environment. The essential difference is that environmental influences on the phenotype are not inherited unless they in addition cause genetic material to mutate. These observations are in part summarized in the epigram: "The phenotype is a product of the interaction of the environment and the genotype." An obvious conclusion derived from the fact that no organism can exist independently of an environment, being itself a segment of the environment. It exists at the expense of its external environment, and is subject to the conditions of this environment. To these conditions it must adapt or die.

1. Modifying the "Normal" Phenotype to the "Mutant" Condition

The "normal" phenotype is that obtained under "normal" environmental conditions under the control of a "normal" or wild-type genotype. Modifications of the environment may cause a departure from what is considered "normal" at one set of conditions to what is considered normal under the new conditions. The extent of phenotypic change is dependent, of course, upon two factors, the degree of environmental change and the response of the organism. This response is determined by the genotype, and different responses to the same types of environmental change will be elicited with different genotypes, as will be discussed in the succeeding section.

Ordinarily the responses of an organism to the usual environmental changes under the control of the observer, such as light, temperature, and food supply, are familiar to the observer as being usual for the organism. There may be changes in growth rate, size, pigmentation, etc., or if the environmental change is drastic, death may ensue. Frequently, these changes (except for death) are reversible. When the environment is returned to its original state, the phenotype accordingly reverts. However, by the employment of special techniques the phenotype of the organism may be so changed, and remain changed, that it resembles specific mutant phenotypes which under normal environmental conditions can only be obtained by a mutant genotype. Mutant phenotypes so produced in genotypically wild-type organisms are called *phenocopies.*

Goldschmidt (208) has produced a number of phenocopies in wild-type *Drosophila melanogaster* with temperature shocks of 35° to 37°C for varying intervals of time, at different stages of the larval period. Many of the adults developing from the heat-treated larvae resemble to a remarkable degree certain known inherited mutant phenotypes. Some of the phenocopies obtained by Goldschmidt are listed in Table 44. Heat is not the only factor which can induce phenocopies. By

Table 44. Some Examples of Phenocopies Obtained in *D. melanogaster* by Treatment with High Temperatures *

Phenotype Induced	Developmental Period Treated, days	Temperature of Treatment, °C	Exposure Time, hours	Per Cent of Phenocopies
Scalloped	4½–5½	35	12–24	70
Curly	6–7	35–37	18–24	76
Spread	5½	35	18–24	91
Trident	7	35–37	6–24	82

* From Goldschmidt (208).

means of sublethal doses of cyanide, silver salts, quinone, and derivatives given the larvae in the medium, Rapoport (502, 503) was able to induce non-inherited phenotypic modifications in the adults which again were strikingly similar to inherited mutant phenotypes known to be dependent upon single gene mutations.

Genetically determined mutant phenotypes may also be modified in the wild-type direction. Many examples of this have already been

given in the descriptions of the responses of nutritional mutants to the compounds required, and in the modification of eye color in mutants of *melanogaster* by transplantation of normal tissues or the addition of an active compound to the food medium. Partial starvation of the homozygous *vermilion* larvae of *melanogaster* which ordinarily do not produce brown pigment results in the synthesis of considerable amounts of this pigment and hence a wild phenotype (36, 636).

These examples serve to emphasize the tremendous plasticity of the phenotype, and show that it must be defined in terms of both genotype and environment. Further emphasis to this important generalization can be best had by a consideration of certain peculiar mutant conditions which are extremely sensitive to environmental factors, particularly temperature, much more so than the "normal" mutant and wild-type conditions.

2. Temperature-"Sensitive" Alleles

The degree of pigmentation of many mammals is considerably affected by the temperature conditions under which they develop and live. Familiar examples of this are certain species of rabbits, weasels, etc., which change from dark to light pelage with the advent of cool weather. The mechanism involved is undoubtedly complicated and related to changes in endocrine activity during different seasons of the year. Generally a change of temperature for a short period has little or no effect on the pelage of most mammals, but only gradual and prolonged changes bring about the condition alluded to above. However, the domestic rabbit has a color variety known as the "sooty" or Himalayan strain which, unlike the other varieties, is extremely sensitive to temperature conditions. When raised at "room" temperature (ca. 22°C) the Himalayan rabbit develops a white pelage with melanic pigmentation in the extremities: the ears, forepaws, tail, and nose darken while the rest of the body remains albino (Fig. 84). The degree of pigmentation is variable in these parts, unless the temperature is kept relatively constant. When the skin in any part of the body is cooled to temperatures under 34°C, melanin production results in that part. If the temperature is raised above this in any part including the extremities, the hairs developing from that part are free of pigment (Fig. 84) (129). Thus the characteristic Himalayan pattern resulting when the rabbit develops under ordinary temperature conditions is a consequence of the temperature in the extremi-

ties being lower than in the other parts of the body which, having a better blood supply, are normally kept above 34°C. A heteronomous condition exists under control of the temperature, rather than an autonomous pattern-controlling agent which is the more usual situation in mammalian pigmentation.

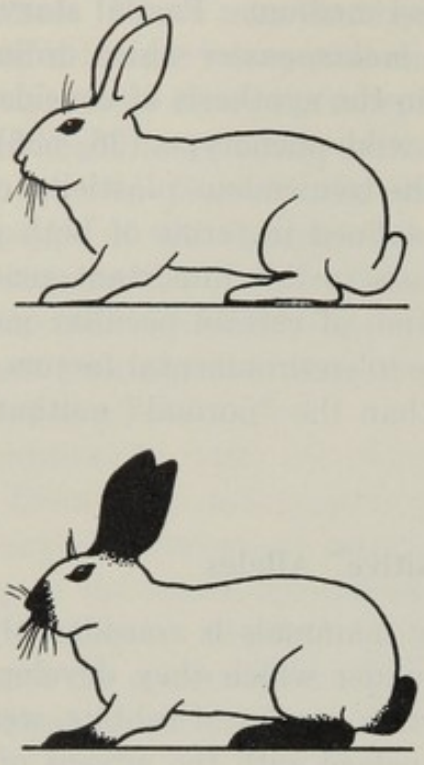


Fig. 84. The phenotypic appearance of Himalayan rabbits under different temperature conditions. *a.* A rabbit raised at a temperature above 30°C. *b.* A rabbit raised at a temperature about 25°C. *c.* A rabbit which has had the left flank artificially cooled at a temperature below 25°C. After Danneel (129).

The Himalayan strain differs from the other color varieties by a single gene, a^n, an allele of the albino series. This series, quite comparable to the *C* series of the guinea pig and mouse previously discussed, includes four alleles, *A*, full pigment; a^{chi}, chinchilla; a^n, Himalayan; and *a*, albino. Only animals homozygous for a^n or heterozygous for a^n/a respond to temperature changes.

Enzyme experiments with extracts of Himalayan rabbit skin indicate that there are at least two phases or reactions involved in the production of the melanic pigment (129). The first phase proceeds under anaerobic conditions and only if the temperature is below 34°C. A temperature of 25°C is optimal. This phase may be associated with the production of an enzyme which converts precursors into melanin in the second phase. The second phase results in melanin production and requires oxygen as might be expected. According to Danneel (129) it is the first, or anaerobic, phase which is different in the Himalayan strains in so far as it is temperature-sensitive to a degree not obtainable in extracts from the other strains. There seems to be a direct relation between the a^n allele and the production of the enzyme, since active extracts from a^n/a^n animals are about 30% more active than those from a^n/a animals.

A similar effect of temperature on the synthesis of an enzyme catalyzing the synthesis of melanin has been found in *Neurospora* by Horowitz and Sheng (296). Little or no tyrosinase activity is found in certain strains of this organism at 35°, whereas at 25° active enzyme is present. An analysis of various wild strains of *Neurospora* has also shown that they differ in the thermostability of the tyrosinase which they contain at 25°. This thermostability difference has been shown to be inherited through a pair of allelic genes and thus constitutes another example of an apparent protein difference brought about by the change in a single gene (292).

A considerable number of the nutritional mutants of *Neurospora* are sensitive to changes in temperature. They require the addition of growth factors to the minimal medium at certain temperatures, but not at others. Figure 52 gives some data for a temperature-sensitive riboflavin mutant which show that in the absence of riboflavin the mutant growth approximates that of wild type under 25°C, but above this temperature growth is sharply reduced to zero at about 28°C. The addition of riboflavin stimulates growth at temperatures above 25°C, and the wild-type level of growth is attained by the addition of 5 μg of riboflavin per 20 ml. Despite the riboflavin requirement the mutant synthesizes riboflavin at temperatures above and below 28°C. But at temperatures above 28°C it is necessary to initiate growth with a small amount of riboflavin (ca. 0.3 μg/ml). The mutant then grows intermittently. It would seem that the production of riboflavin and its utilization are the limiting factors. At temperatures above 28°C the mutant cannot both synthesize riboflavin and proliferate at the same time.

The presumed allelic series of pyrimidine mutants of *Neurospora* described by Houlahan and Mitchell (302) includes two temperature-sensitive alleles (Table 45). Strains 37815 and 67602 both require

Table 45. Growth Requirements of Three Allelic Strains of *Neurospora crassa* for Pyrimidine at Two Different Temperatures *

	Milligrams of Hydrolyzed Ribonucleic Acid per 20 Ml Medium Required for ½ Maximum Growth	
Strain	25°C	35°C
37301	3.3	3.2
37815	0	2.4
67602	0.38	2.3

* From Houlahan and Mitchell (302).

a preformed source of pyrimidine at a temperature of 35°C, but at 25°C 37815 grows without pyrimidine whereas 67602 requires much less than at 35°C. An allelic strain, 37301, requires pyrimidine at the same concentration for growth at both temperatures.

Many of the known mutant genes in *Drosophila* have a very variable expression. This is particularly true if the character has only partial penetrance as in the *cubitus interruptus* example mentioned

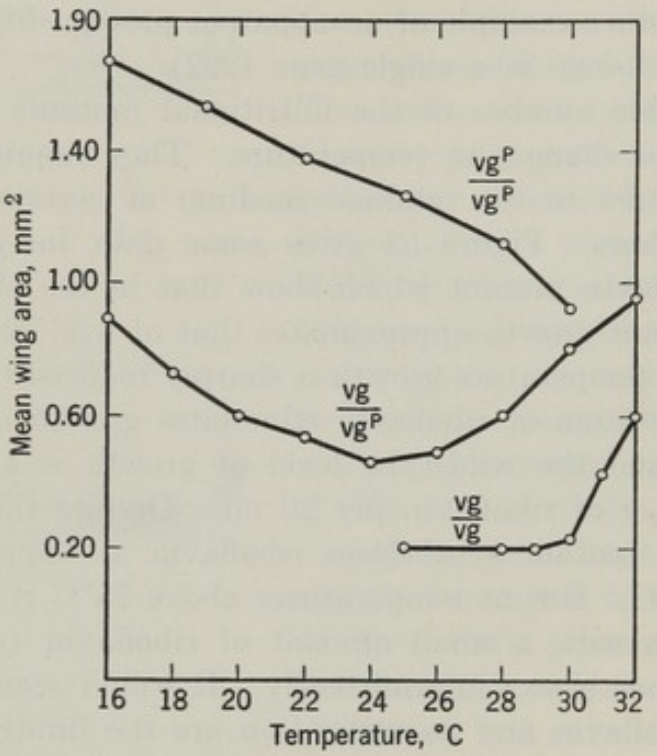


Fig. 85. The effect of temperature on the area of the wings in vg^P and vg *Drosophila melanogaster*. After Harnly and Harnly (258). Data for males only.

previously (p. 230). Failure to control rigidly the temperature during the course of experiments involving the *ci* series would result in great variations in phenotypic ratios from one experiment to the next. The vestigial series of alleles (see p. 287) also has members which have a temperature-labile phenotypic expression. Harnly (256, 257, 258) has conducted extensive investigations into the effects of temperature on the expression of the alleles *vg* and vg^P (pennant). Figure 85 describes the results obtained by raising flies of the genotypes vg^P/vg^P, vg^P/vg and vg/vg at temperatures ranging from 16° to 32°C. The effect of each genotype was determined by measuring the wing area. The expression of homozygous pennant closely parallels that of wild type in the reduction of wing area over the temperature range indicated in the figure, and in this sense vg^P is not any more tempera-

ture-sensitive than vg^+. But when vg^P is heterozygous with vg a marked temperature effect is demonstrated. Homozygous vg is modified only at temperatures above 29°C under which conditions vg/vg flies approach the wild phenotype. The range is a narrow one, however, for above 32°C the homozygous vg flies do not develop. A corresponding lethal effect of temperature in a range which is not lethal for wild type is also noted in vg^P/vg^P flies. These will not develop at temperatures above 30°C. Similar extreme temperature-modifying effects have been noted in *Drosophila* for the *Bar* mutants (272) and the *white* allele, *blood* (w^{bl}) (171). (See Fig. 88.)

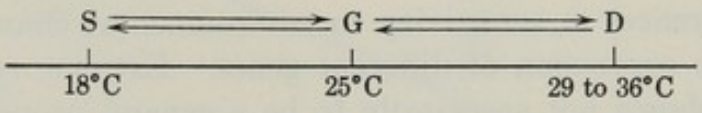
These observations and experimental results clearly establish the great differences in sensitivity to environmental change shown in the phenotypic expression of different genes. Extreme sensitivity is furthermore shown not necessarily to be a general property of groups of allelic genes related by their effect on the phenotype, but of particular genes within each allelic series.

3. Serotype Transformation in *Paramecium aurelia*

When paramecia are injected into a rabbit, the rabbit reacts with the production of antisera which immobilize the strain used as the antigen source. Apparently the active antigens are ciliary in origin, for the antisera cause a paralysis of ciliary motion. Sonneborn (572, 573) and Beale (37) were able, by the establishment of homozygous stocks of *Paramecium aurelia,* to demonstrate that a given homozygous strain apparently produces only one type of ciliary antigen at a time. The particular antigen produced depends upon the conditions of culture and the previous history of the stock. For example, Sonneborn has shown that stock 51 of *Paramecium aurelia,* variety 4, which arose from a single homozygous individual, exhibits any one of a number of antigenic types, or *serotypes,* designated as A, B, C, D, E, G, H, and S. A culture of any one of these serotypes may maintain its antigenic specificity through many vegetative fissions (or cell divisions), and through conjugation, provided the growth conditions are maintained relatively constant and optimal for the particular serotype with respect to temperature and nutrition. Serotypes A, B, and D of stock 51 were maintained for over four years at 26° with no change (575). However, by a number of different environment modifications such as (1) change in temperature, (2) change in nutrition, (3) subjecting the serotype to sublethal doses of its specific antiserum, and (4) exposure to ultraviolet light (573), it is possible

to induce *transformations in serotype.* Thus serotype A can be transformed to B by lowering the temperature to about 19°. Once established, serotype B is constant provided the proper conditions of culture are maintained, but the transformation is reversible, for B may be induced to change back to A. Breeding experiments have demonstrated conclusively that no genic changes accompany the transformations, but apparently paramecia of the same genotype can be of any one of the seven serotypes enumerated above.

Four homozygous stocks of *P. aurelia,* variety 1, collected from different parts of North America, have been intensively studied by



Stock			
90	(d g^{90})s	(d)g^{90}(s)	d(g^{90}s)
60	($d^{60}g^{60}$)s	(d^{60})g^{60}(s)	d^{60}(g^{60}s)
41	($d^{41}g^{41}$)s	(d^{41})g^{41}(s)	d^{41}(g^{41}s)
61	(d g^{61})s^{61}	(d)g^{61}(s^{61})	d($g^{61}s^{61}$)

Fig. 86. The serotypes produced at different temperatures in *Paramecium aurelia.* After Beale (37).

Beale (37) with a view to extending the original observations of Sonneborn on variety 4 partially described above. The variety 1 stocks, 90, 60, 41, and 61, have each been marked with three different loci identified phenotypically by the different serotypes S, G, and D. The particular antigenic condition demonstrated by a culture of any one of these stocks is dependent upon the temperature. At 18°C the serotype of a population is predominantly S; at 25°C, G; and at 29° to 36°C, serotype D (Fig. 86). The transformations are for the most part easily reversible, but occur only after a number of fissions have taken place at the new temperature. In stock 90, serotype G is transformed to D when the temperature is raised to 29°, but is complete only after 50 fissions (10 days). The transformation itself is relatively sudden, taking place within the space of two fissions. Prior to this the serotype is G. During the short transition the cells react to G and D antigens, but when transformation is completed only D antigens are effective. As demonstrated by Sonneborn with variety 4, the serotypes are mutually exclusive, both cannot be maintained simultaneously except during the short period of shifting from one stable antigenic state to another. Serotype D can be caused to re-

vert back to G in stock 90 by lowering the temperature to 25°C. Transformation is complete after 11 fissions (3 days).

The corresponding serotypes of the four homozygous stocks are designated by identical letters because they induce heterologous antisera which cross react in varying degrees with each other's antigens. Hence the S antigens, 90S, 60S, 41S, and 61S, all induce antisera which cross-react with the S serotypes of all four stocks. Indeed the S antigens of stocks 90, 60, and 41 cannot be serotypically distinguished. 61S antibody cross reacts but only at low dilutions. The D antigens of stocks 90 and 61 appear to be identical, those of 60 and 41 dissimilar but capable of cross reacting with one another's antibodies. The G antigens show only weak cross reactions among the four stocks and are hence presumed to be all four different.

As the result of breeding tests in which recombinations were obtained in crosses between different stocks, and in which heterologous antigen production segregated according to Mendelian expectations, Beale has postulated the existence of three genes in each stock. Each gene is concerned with the production of a different antigen. Thus stock 61 is genotypically, d/d, g^{61}/g^{61}, s^{61}/s^{61}. The serotype expressed is dependent upon the conditions expressed above. In Fig. 86 the genotypes for the four stocks are given by way of summary. Heterologous antigens are designated by allelic genes, because of the result obtained from crosses. The parentheses enclose those genes which are presumably not expressed at the temperature indicated. Nearly all possible recombinations have been obtained from crosses between the different stocks. Those with recombinations demonstrate the same ability to change serotype, but the specific serotype shift will be different than that found in the original homozygous stocks. For example, the F_2 from a cross between 90G $\times$ 60G produces recombinations of the genotype $\frac{d^{60}g^{90}s}{d^{60}g^{90}s}$. At 25°C these will show the serotype 90G, but at 29°+ the serotype will be 60D, not 90G.

Figure 87, *a* and *b*, presents further results from crosses between stocks which illustrate the effects obtained in heterozygotes. If 90G is crossed to 60G (37), the F_1 hybrids after several fissions at 25°C demonstrate both serotypes, 90G and 60G. It is interesting that the effect of the immobilizing antisera—90G and 60G—is less marked on the heterozygotes than on the respective homozygous parents. This fact may be interpreted as meaning that the same amount of antigen of general type G is produced in the heterozygotes as in the homozygotes, but less of each specific type. Here again is another piece

of evidence for the generalization that the amount of gene product is related to gene dosage. By causing the heterozygotes to undergo autogamy, a kind of internal sexual reproduction (see Diller (141); Sonneborn (571, 574)) which results in homozygosity, animals with

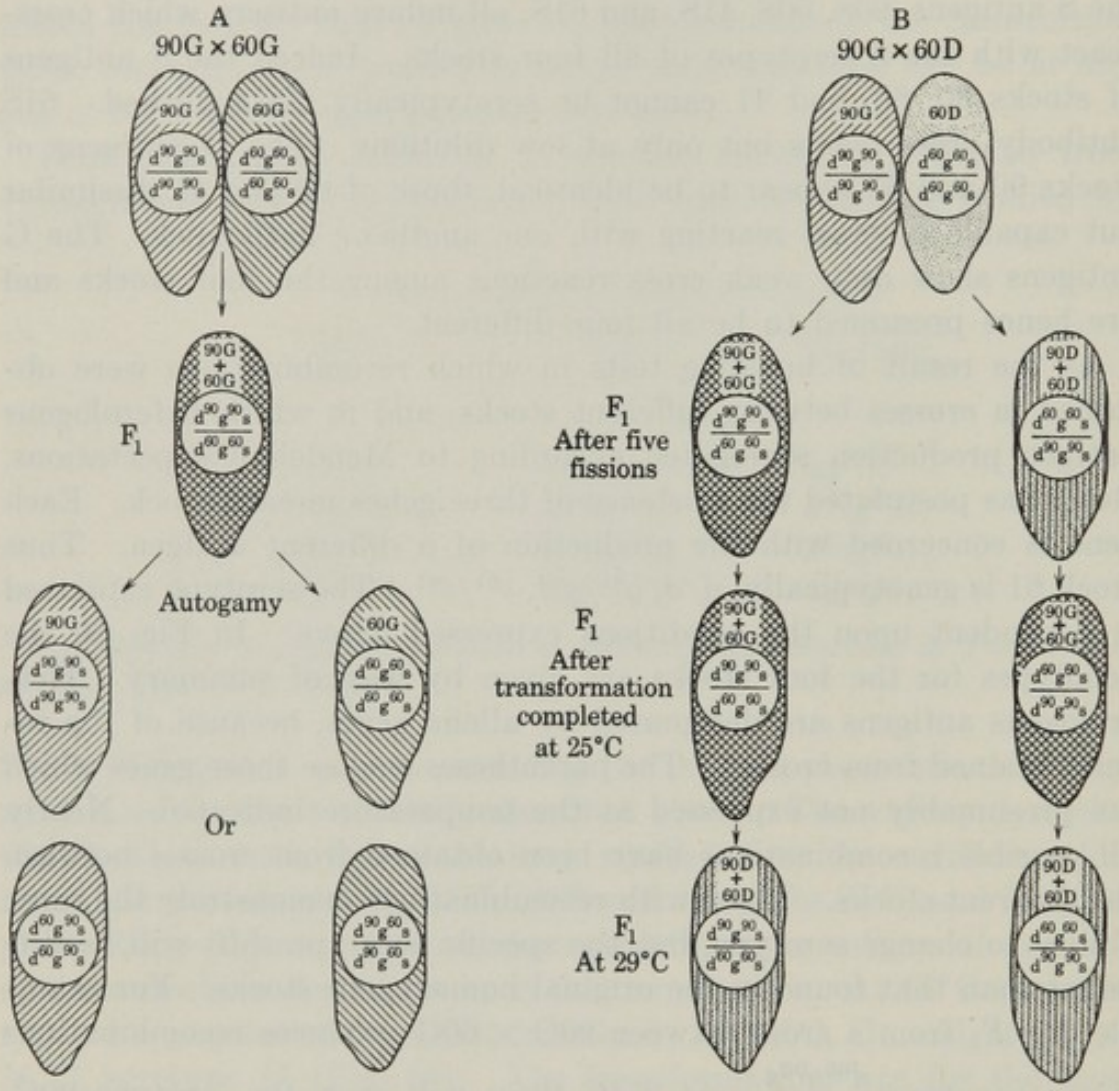


Fig. 87. The results of crosses between paramecia of different serotypes. After Beale (37).

either 90G or 60G serotypes, but not both, are obtained. It is, incidentally, in these exautogamous individuals (F_2) that recombinations will be detected.

Since 50 or more fissions may be required to transform serotypes, crosses may be made between individuals of different stocks and serotype as illustrated in Fig. 87*b* with a cross between 90G and 60D at 25°C. The heterozygotes produced here show as expected both 90 and 60 stock traits, but the initial individuals derived from the orig-

inal conjugant with G antigen are 90G + 60G whereas those from the other parent are 90D + 60D. After, however, a more prolonged period at 25°C the transformation is completed and only the G serotype is expressed in all individuals. This observation is of great interest, for it demonstrates the principle that the phenotypic expression of a gene is dependent upon the immediate environment. Gene d^{60} removed from the 60D cytoplasm and transferred into 90G cytoplasm is not expressed, but g^{60} is. Gene d^{60} expresses itself only when the temperature is raised to 29° (Fig. 87*b*, bottom row).

By a combination environmental and genetic analysis Sonneborn and Beale have provided a crucial test for the role of genotype and environment. They have demonstrated conclusively that the phenotype is defined by both even for so important an aspect of the organism as its antigenic structure. In addition they have provided important information relating to the general problem of the relative roles of cytoplasm and genes. Obviously the cytoplasm is subject to change by the environment, but most important is the fact that there are limits to the degree and kind of change which are dictated by the nuclear genes.

4. The Temperature-Sensitive Period

The phenocopies obtained by Goldschmidt, as described on p. 290, were induced by the application of temperature shocks of different duration, and at different times during the larvae period as shown in Table 44. The time of application and the duration of application of the shocks for the maximum production of the different phenocopies seem to be different. For example, a *miniature* phenocopy is produced optimally by treating old larvae whereas *scalloped* phenocopies are best induced by treatment of somewhat younger larvae. This would indicate a *sensitive period* in development, at which time a particular abnormality is most easily produced by interfering with a specific phase of development. Changing the time of application of the shock would be expected to interfere with a different phase of development and hence produce a different phenocopy.

A specific sensitive period is particularly evident in the influence of temperature on the phenotypic expression of *vestigial.* The modification of the vestigial phenotype in *vg/vg* flies caused by temperatures of 30° to 32°C is most extreme when larvae otherwise raised at optimal temperatures (ca. 25°C) are subjected to elevated temperatures at the beginning of the third larval instar (about 64 hours

after hatching) (256, 258). Temperature shocks applied prior to the third instar have no effect on the expression of *vg*. Hence the third instar is a sensitive period with respect to the expression of *vg*.

The *white* allele, w^{bl}, also has a temperature-sensitive phenotypic expression with a definite time of induction (171). Homozygous, w^{bl}, flies have pale brown eyes at 30°C and deep red-purple eyes at 17°C. Both the brown and red pigment components are affected (Fig. 88). The temperature-sensitive period occurs during the pupal stage about 40 to 48 hours after the onset of pupation (Fig. 88). Treating larvae of any stage, or young or old pupae, at low or high temperature has no effect on the eye color of flies raised at 25°C.

The occurrence of sensitive periods in the development of multicellular, differentiated organisms during which certain phenotypic modifications are most easily produced is a significant phenomenon which will be considered further in Chapter 13. Precisely the same phenomenon is observed by the experimental embryologist who effects changes in the final form of an organism by altering the course of development of the embryo with chemicals, transplantations, and physical injury. Various types of abnormalities may be produced, depending on the stage of development subjected to treatment. The genetic observations, however, add to the embryological observations in showing that the degree of sensitivity of phenotypic response to stimuli during certain periods of development is markedly altered by the genotype.

Goldschmidt (208) attempts to equate the observed effect of the environment at specific periods of development to produce phenotypic alterations with the action of the genes which appear to control the particular phenotypes. In connection with his analysis of phenocopies he concludes that ". . . the processes underlying the formation of phenocopies are the same as those set in motion by the mutant genes." This is an elaboration of his "rate concept" which states that the role of genes is the regulation of rates of reactions and hence development. There is no doubt that genes, like temperature, control the rates of reaction through their undoubted effects on enzyme production and activity, but there is complete ignorance of the mechanism by which they do this. Therefore it is not justifiable to conclude that the processes involved in the modification of phenotype by environment are identical to those produced by gene mutation.

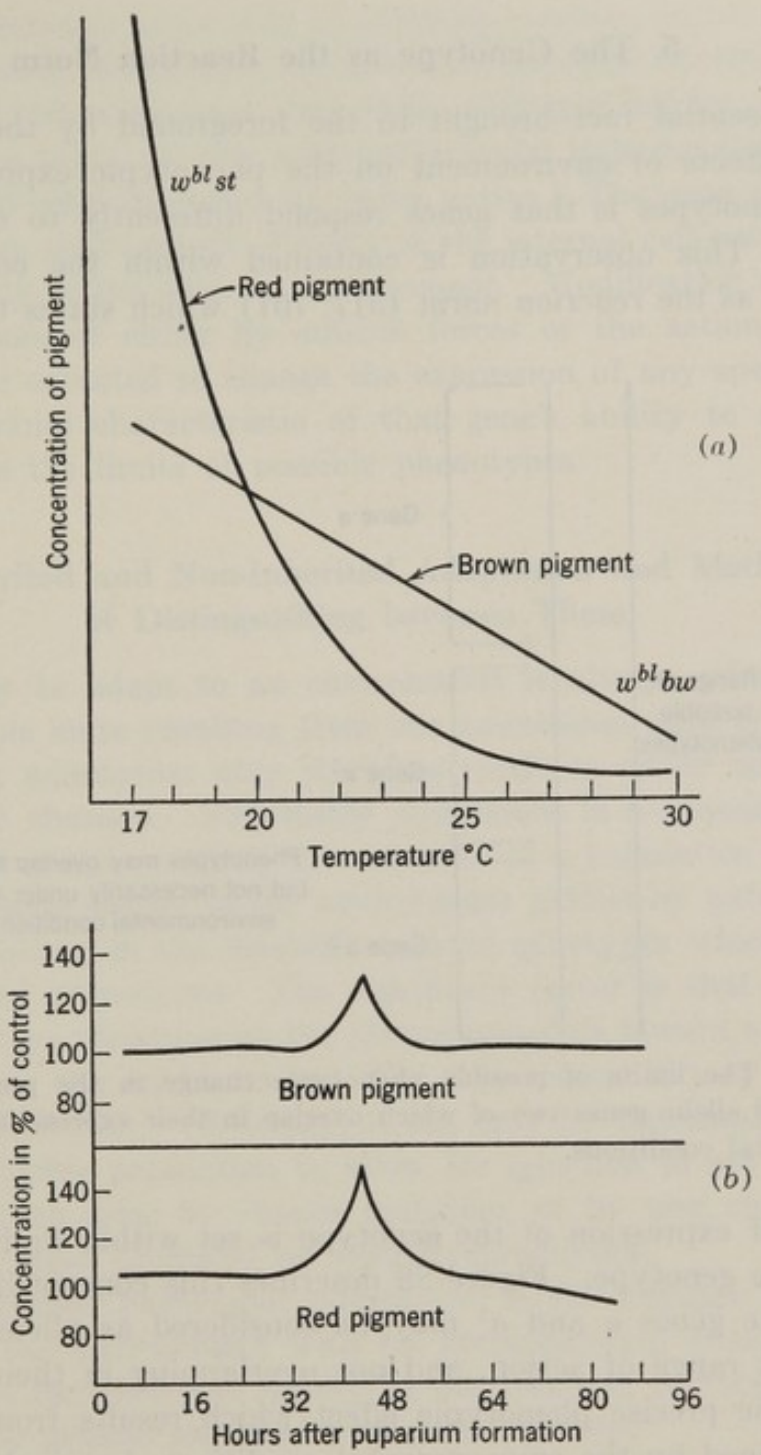


Fig. 88. The effect of temperature on the production of red and brown pigment in *Drosophila melanogaster,* homozygous for w^{bl}. After Ephrussi and Herold (171). (*a*) The concentration of pigments in adult *blood* flies raised at indicated temperatures. (*b*) The concentration of pigments in adult *blood* flies raised at 25°C but subjected for 8-hour-periods to a temperature of 17°. Note that the sensitive period for pigment production is between the 40th and 48th hour of pupal development.

5. The Genotype as the Reaction Norm

The essential fact brought to the foreground by the experiments on the effects of environment on the phenotypic expression of different genotypes is that genes respond differently to environmental stimuli. This observation is contained within the concept of the genotype as the *reaction norm* (317, 701) which states that the mod-

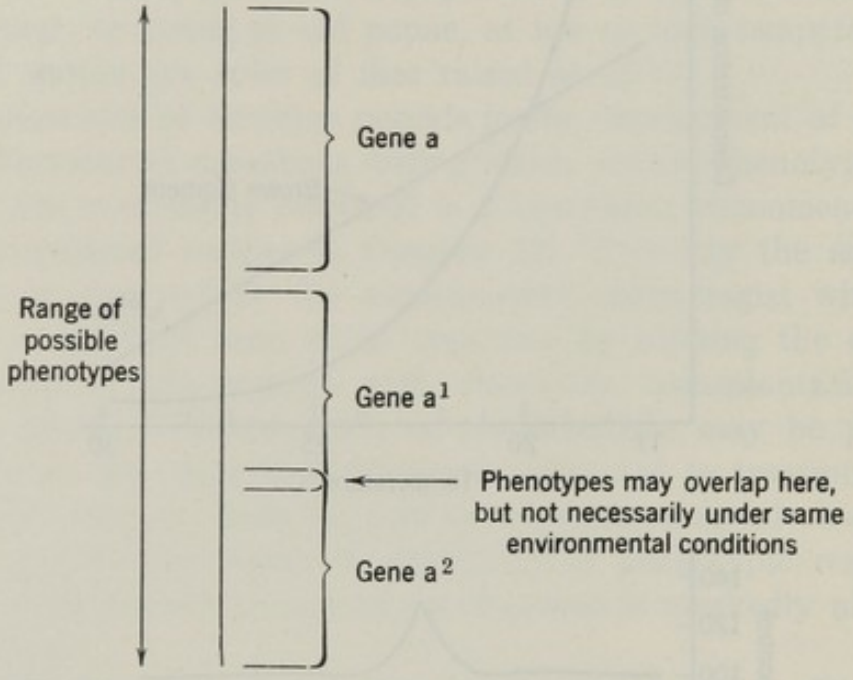


Fig. 89. The limits of possible phenotypic change in the presence of three hypothetical allelic genes two of which overlap in their expression under certain environmental conditions.

ification of expression of the genotype is set within limits characteristic of the genotype. Figure 89 describes this concept diagrammatically. The genes a and a^1 may be considered as alleles, each with a different range of action, and not overlapping in their phenotypic effect. The precise phenotypic effect which results from either will be determined by the environmental conditions, but under no known conditions will the phenotypes overlap. Actual examples to fit these conditions may be found in the many allelic genes which are autonomous in their expression and show no overlap of phenotype regardless of the environmental conditions. On the other hand, gene a_2 overlaps with a_1 in its expression range, and under certain environmental conditions their phenotypic expression will be identical. Isoalleles such as ci^{+2} and ci^{+3} of the *cubitus interruptus* series (p. 238)

may be considered as genes with overlapping reaction norms, as should any mutant genes which under certain environmental conditions overlap in phenotypic expression with their wild-type alleles.

The reaction norm concept is of fundamental importance to an understanding of what is meant by "gene action." The gene never acts alone but in an environment, directly in the internal cell environment, more indirectly in the external environment. Modification of the internal environment either by outside forces or the action of other genes may be expected to change the expression of any specific gene within the limits characteristic of that gene's ability to act. The genotype sets the limits of possible phenotypes.

6. Inherited and Non-Inherited Adaptation and Methods of Distinguishing between Them

The ability to adapt to an environment is always inherited, but the phenotypic state resulting from the adaptation may or may not be inherited; adaptation may therefore result in either inherited or non-inherited changes. Inheritable adaptation is a *population* phenomenon. It is the result of the response of a population of organisms of the same species to the environment guided by natural selection and resulting in the establishment of genotypes which produce the best fitted phenotypes. The significant factor is that *change in genotype* always accompanies the phenotypic shift toward a state better adapted to the environment. For this reason it may be called *genotypic adaptation.* Obviously, it can only be expressed in an actively reproducing population to allow for selection to act upon new genotypes which arise by chance mutation, or by new chance combinations of genes produced by sexual reproduction.

Non-inheritable, *phenotypic adaptation* is the response of the *individual* to the environment with no accompanying changes in the genotype. It can therefore hardly be the result of mutation and selection of the genetic elements within the nucleus.

Ordinarily the distinction between the two meanings of adaptation is easily resolved, and it may seem that the academic hair is being too finely split in the above statements. When large organisms such as corn plants or fruit flies are being dealt with, the question of the origin of individual phenotypic changes can easily be answered by breeding experiments. But a closer scrutiny of the distinction becomes mandatory when dealing with microorganisms, since populations of independent single cells or nuclei produce the observed phe-

notype as a unit. Unless the experimenter is able to observe and breed a single cell, it becomes necessary for him to establish the type of adaptation resulting in phenotypic change by methods of analysis which may be quite circuitous.

If the microorganism is one which can be carried through a sexual cycle, variants which appear can be tested by crossing them to the original, unadapted strain. An example of this direct approach may be found in the adaptation of a pantothenic acid-requiring mutant of *Saccharomyces cerevisiae* described originally by Lindegren and Lindegren (380). If cells of this strain are inoculated into a medium completely deficient in pantothenic acid, they may remain viable for a month or more and accomplish so little growth that no visible turbidity appears in the tubes (505). During this period, however, some cells develop the capacity to synthesize pantothenic acid, multiply, and within a few days cause a definite turbidity. Genetic analysis of crosses between the adapted cells and the original unadapted strains shows that the spores resulting from the cross segregate in a one to one ratio of pantothenate dependent to pantothenate independent, proving that the adaptation is due to mutation to ability to synthesize this compound and subsequent selection in a pantothenate-deficient medium. All the pantothenate independent cells that have been tested by Raut (505) have also been determined to be different from the original strains of *S. cerevisiae* which are able to synthesize pantothenic acid. The reversion mutations therefore were probably suppressors of the pantothenicless gene in the unadapted strain.

Several conclusions may be derived from the above analysis which emphasize the importance of considering the origin of adaptation by mutation and selection in any experiments concerned with phenotypic change in microorganisms. First, cells of some organisms may remain viable for long periods with only slight growth, in a hostile environment; in the example given, a deficient medium. Second, given the time provided by this extended viability, the chances of mutations providing for synthesis of the absent and required compound are increased. Third, the mutation may not necessarily be a simple reverse mutation to an identical gene type present in all synthesizers, but may be of the suppressor type. Thus chances for a mutation to adaptation to a deficiency are theoretically increased by the possibility of mutation at a number of different loci all resulting in essentially the same effect.

It is also to be noted in this connection that genotypic variation may arise by mutation in a population, and the phenotypic effects be masked by the phenotype of the original dominant genotype which may be best fitted to the environment. The variants, if they are nutritional mutants, for example, can easily maintain themselves by having their nutritional requirements satisfied by the strains which produce the required compounds and excrete them into the medium. A comparable condition is well illustrated by fungi such as *Neurospora* which being essentially coenocytic can exist as stable heterocaryons with a population of diverse nuclei sustaining one another's capacities symbiotically. If the environment changes, the relationship between the different genotypes may change by selection and cause a shift in the phenotype, an event which would be termed an adaptation.

These observations make it clear that, despite the random nature of mutation and the rareness with which it may occur for any particular gene in the direction of fitness to the environment, adaptation in populations of cells or nuclei, as in *Neurospora* by mutation and selection, is to be regarded as a highly probable explanation whenever a shift in phenotype is noted.

When dealing with the asexual strains of bacteria frequently used in adaptation studies, the technique of breeding cannot be applied and other methods must be relied upon to distinguish between phenotypic and genotypic adaptation. Some evidence that an observed adaptation in these forms is of the purely phenotypic variety may be had if the adaptation is a rapid one, i.e., within a span of a few hours, for one would not expect adaptable variants to increase in numbers sufficient to be recognized in so short a time. However, such a method is of no value if the adaptation is a slow one.

A second type of evidence which may point to phenotypic adaptation is a positive capacity to deadapt to the original phenotypic condition in the presence of the original environment. This again, however, is not critical evidence that phenotypic adaptation and not mutation has occurred. An actual example taken from the work of Ryan and Schneider (532, 533) will make this clear. These workers have shown that a strain of *E. coli* which requires histidine readily adapts to growth on a medium deficient in histidine, and once adapted just as readily deadapts to the original requirement when cultured in the presence of histidine. The use of refined plating techniques has shown that the adaptation to histidine independence on histidine-free medium is due to mutation and selection, and that the mutation rate

among the mutants back to the original dependent condition is sufficiently high to provide for the presence of enough of them to account for deadaptation. There appears to be a definite inhibition of growth of the histidine independent cells when in the presence of those dependent on this compound. This unexpected relationship leads to very rapid growth of the histidine dependent mutants on histidine-containing media and to the illusion of deadaptation of the purely phenotypic variety.

A short period for adaptation and capacity to deadapt are both *prima facie* evidence for phenotypic adaptation, but neither constitutes critical proof. They must be supplemented by additional tests which are designed to detect adaptive capacity in the absence of factors which might select for or against the changed phenotype. Such a test has been devised by Luria and Delbruck (385) and used successfully by them and numerous others to distingiush between mutation and simple adaptation in microorganisms. The test is so designed as to detect the origin of adaptable strains in the absence of the adapting substrate, an event which, if it occurs, can only be assumed to be the result of mutation.

Perhaps the most direct method of elucidating the cause of adaptation when dealing with microorganisms is to observe whether phenotypic change occurs in a non-proliferating culture. Such changes as then arise (for example the appearance of a new enzyme activity in the presence of a new substrate) cannot be due to mutation unless all cells in the population mutate to the same condition—a highly unlikely occurrence. This method cannot always be applied since many true phenotypic adaptations in microorganisms will not occur unless the culture is growing. In this case the other methods described above must be resorted to.

7. Enzymatic Adaptation

One of the most interesting, and perhaps most significant from the biochemical point of view, of the phenomena of phenotypic adaptation is that of enzymatic adaptation in which an organism produces a previously undectectable specific enzyme activity in the presence of a substrate attacked by that enzyme. Figure 90 describes the adaptation of a yeast, *S. cerevisiae,* in the presence of galactose to ferment galactose. Ordinarily, *cerevisiae* is grown on a substrate containing glucose as the available carbohydrate, and it accordingly ferments this sugar readily as shown in Fig. 90. When cells grown on

glucose are washed free of glucose and placed in a nitrogen-deficient medium containing galactose they do not reproduce in numbers but after a lag of a few hours begin to ferment galactose. The lag period is one in which the cells presumably shift from a glycolytic mechanism tuned to glucose to one adapted to galactose by the formation of a "galactozymase." The following facts prove that the phenomenon is an adaptation of the cells' systems to utilization of a specific substrate, galactose, and not a selection of mutants which

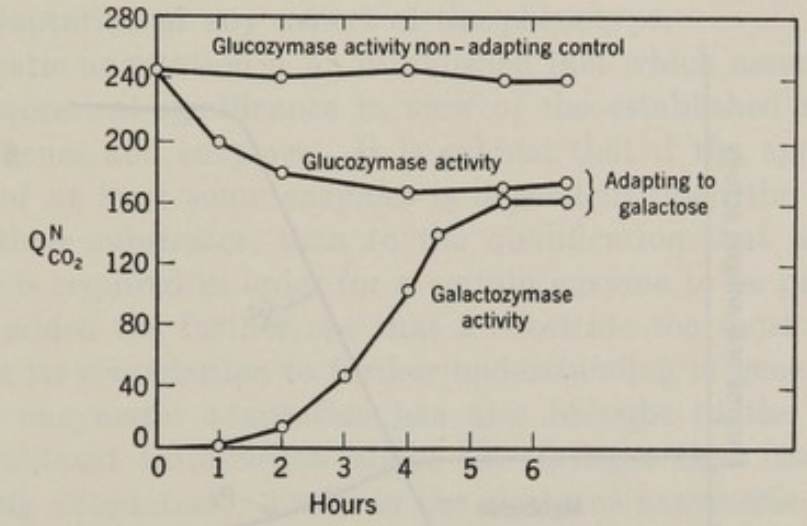


Fig. 90. The adaptation of yeast to the fermentation of galactose while in the presence of galactose. After Spiegelman and Dunn (583).

arise from glucose-fermenting cells. (1) The adaptation occurs in resting cell suspensions in which the cells are prevented from reproducing by the absence of available exogenous nitrogen. (2) The adaptation occurs only in the presence of galactose. (3) Resting cell suspensions which are adapted to the utilization of galactose readily deadapt in the presence of glucose, and can therefore only ferment galactose again after the usual lag period.

Of the several possible explanations for the underlying cause of this example of phenotypic adaptation in yeast, two seem the most plausible. (1) The cells respond to the presence of galactose by producing a specific enzyme or set of enzymes, collectively described as galactozymase, necessary for the fermentation of galactose. (Actually galactozymase is two or more enzymes. See p. 184.) (2) The galactozymase is always present, but the cells are impermeable to galactose after growth on glucose and must during the lag phase readjust their permeability to admit galactose. That the second explanation is not correct and the first more plausible is shown by the

demonstration that cell-free extracts of cells raised on glucose have no galactozymase activity whereas galactozymase is found only in the juice of those previously adapted to galactose.

Further evidence that an adapting enzyme increases in activity through synthesis from precursors without activity rather than merely by uncovering the activity of enzyme already present is to be found in the studies of adaptation of *E. coli* and to β-galactoside hydrolysis (113). In the presence of melibiose *E. coli* produces a measurable β-galactosidase activity in less than an hour (Fig. 91). Ac-

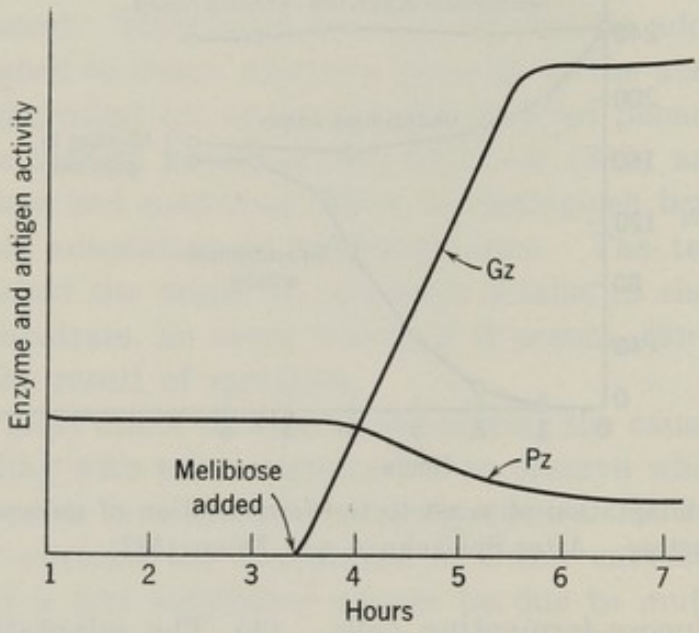


Fig. 91. The adaptive formation of β-galactosidase in *E. coli*. After Cohn and Torriani (113).

companying this increase in activity is a demonstrated equivalent increase in an antigen, Gz. The antigen is either the enzyme or closely bound to it, for a highly purified preparation of the enzyme induces the formation of an antibody which specifically combines with the enzyme from adapted cells. Figure 91 gives data showing the close correspondence between the increase in enzyme activity and antigen titer. Since there is no indication that unadapted cells have Gz antigens, these data can be taken as proof that protein synthesis is involved in enzymatic adaptation in this example.

Numerous other examples of adaptation which are best interpreted as being the result of response to a given substrate by producing an enzyme specific for that substrate are known in microorganisms. (See Monod and Cohen (435).) In some instances the appearance of enzyme activity in the presence of the substrate occurs only in grow-

ing cell suspensions. However, mutation and selection have been ruled out in many of these by the methods of analysis described in Section 6. Not all workers have subjected their experiments to the discipline of genetic analysis, and many so-called examples of enzymatic adaptation described in the literature are unclassifiable as mutation or phenotypic adaptation owing to the lack of suitable experimental control to distinguish between the two possibilities. The power of natural selection and the incidence of mutation in large populations are factors which, as stressed above, must always be considered in analyzing adaptation of any aspect of the phenotype.

Enzymatic adaptation is an established fact which assumes considerable theoretical significance in view of the established relationship between genes and enzymes. It is evident that if the appearance of activity of at least some enzymes is dependent upon the prior presence of their substrates, then to the qualification that a particular genotype is required in order for a certain enzyme to be present there must be added the further one that a substrate too must be present.

Besides its contribution to further understanding of gene action, the study of enzymatic adaptation has also brought to the foreground some significant information about the changes that occur in cells undergoing adaptation. Early in the study of enzymatic adaptation it was recognized that substrate utilization in the simultaneous presence of a number of different substrates with equivalent metabolic functions, such as energy-yielding sugars, was not necessarily simultaneous (140). For example, in the presence of both glucose and sorbitol, *E. coli* utilizes the glucose first, and then adapts to the utilization of sorbitol (434). The growth curve obtained shows an initial period of growth due to utilization of glucose alone, then a lag, and finally a second period of growth during which time sorbitol disappears from the medium. The two-step character of the curve has resulted in the name *diauxie* being applied to the general phenomenon.

Originally the interpretation had been that one of the substrates directly inhibited the utilization of the other, but there is no direct evidence for this. Another obvious possibility is that intracellular competition for available amino acids and protein with which to synthesize enzyme is responsible. Spiegelman (583) in connection with the experiments described on page 307 involving galactozymase activity showed (Fig. 90) that cells adapting to galactose utilization progressively lose a certain amount of their glucozymase activity. This occurred, however, only in the absence of an exogenous nitrogen

source. In the presence of exogenous nitrogen the reduction in glucozymase activity does not take place.

A similar result was obtained by Cohen and Torriani (113) in an experiment utilizing the adaptation of *E. coli* to β-galactoside breakdown. When the β-galactosidase activity associated with antigen Gz increases in cells subjected to melibiose (Fig. 91, curve *A*), there is a simultaneous but not equal decrease in an antigen designated as Pz (Fig. 91, curve *B*). Although no enzyme function has been associated with Pz, it must be considered to have some role to play in *E. coli* as an important cellular constituent. Its decrease, as well as the decrease in glucozymase activity in yeast in the absence of exogenous nitrogen during synthesis of other enzymes, indicates that the available protein of the cell is limited. A corollary possibility should be considered, namely, that the enzyme protein which decreases in amount is structurally related to the one being synthesized, and therefore may provide material directly for its synthesis.

By the elegant use of analysis of changes in free amino acid pools in the cells, and compounds containing ^{14}C, Spiegelman and Halvorson (584) have obtained evidence indicating that enzymes formed during adaptation are synthesized primarily from amino acids in the cells' free amino acid pool, and that protein already present contributes to a quantitatively insignificant extent and only indirectly. Furthermore, it has also been shown by means of ^{35}S tracer experiments that the Pz protein S is not utilized in the synthesis of Gz protein. Hence Pz cannot be considered a precursor of β-galactosidase (284) under the experimental conditions used. On the other hand, investigations by Dubnoff (153) have provided evidence that preformed but enzymatically inactive protein does serve as a reservoir in adaptive enzyme formation if the proper activating system is present. As pointed out by Dubnoff the adaptive enzymes may be formed from both the amino acid pool and from enzymatically inactive but preexisting protein.

8. Conclusions

A consideration of the role of the genotype in producing the phenotype has shown that a living organism consists in part of a coordinated system of genes upon which is superimposed an adaptive system subject both to the genetic system and the environment. The range of response of the adaptive system to the environment, and the manner of its response, are subject to the limitations imposed

by the system of genes. The genetic system controls the elaboration and continuity of the adaptive system which in turn insures the survival and reproduction of the genetic system.

What is the adaptive system? Actually it is the whole organism or the whole cell, but with genotype constant it may be called the extragenic part of the cell. Therefore it is primarily the cytoplasm, although it must be recognized that determinants have been located in the cytoplasm and that the action of a gene may be considerably altered without the gene itself being altered.

These considerations lead to a realization of the importance of the whole cell in the expression of the genotype in the final phenotype. It will be the purpose of the next chapter to inquire into the knowledge of the functions of the cytoplasm as they apply to an understanding of the manifestation of inherited characters as expressed through the whole cell.

The examples of the antigen shift with change in temperature in *Paramecium aurelia,* and the enzyme shift in yeast and bacteria with change in substrate, may be interpreted as illustrating a very important principle with regard to cellular economy. In both of these examples there was noted signs of a competition for available protein. In *Paramecium* one antigenic type always seems to triumph over the other—given proper environment and time. In yeast and bacteria the effect is not so drastic as to result in complete exclusion of one protein type at the expense of the other. But, nevertheless, the presence of one specific type of protein under certain conditions is directly related to the reduction in activity or amount of another. These conditions are to be expected in view of the limitations of the cell as a finite entity with respect to active surface intake and output and available space within to contain the kinds of molecules needed to carry out the life processes. Presumably they are a reflection of the metabolic turnover of the cell being shifted from one approximately steady state to another. Since the available material to turn over is limited, then any change in state should be expected to have widespread effects within the cell.

CHAPTER 12

The Continuity of Cellular Organization

Historically, the chromosomes have occupied a central position in the study of genetics since the beginning of this science. The almost immediate recognition of these cell particles as being the loci of the Mendelian units led to their continued intensive study almost to the exclusion of the remainder of the cell. However, as attention was gradually turned more and more to a consideration of gene expression, it became recognized that any analysis of gene action must take into account the whole cell and whole organism. Furthermore, once a technique was well established for the recognition of inheritance through the nucleus, it became possible by elimination to recognize inheritance through the extrachromosomal parts of the cell. Extrachromosomal inheritance, generally termed cytoplasmic inheritance (although the first term may be more applicable since it includes the nuclear sap and other non-chromosomal elements) was discovered very early in the development of genetics by Correns (117) and Baur (24). Hence we now have two branches of the study of inheritance referred to as chromosomal and cytoplasmic inheritance respectively.

It will be the primary purpose of this chapter to inquire into the role of cytoplasmic inheritance and to attempt to relate the functions of the nucleus and cytoplasm so as to develop a clearer picture of the present understanding of cell function in the individual and in inheritance.

1. The Subunits of the Cell and Their Continuity

It has been stated previously that the cell should be regarded as the unit of life and reproduction (Chapter 2). A statement as broad as this requires some qualification and clarification, first as to what is meant by life unit. Actually there are many different kinds of life units. For example, an isolated, self-sufficient population of animals and plants is a life unit formed of subunits termed species, the species in turn of individuals. If the individuals are multicellular it must be considered that they are in turn composed of subunits, the cells. How far can one continue to break this hierarchy down into subunits? From the purely physical and chemical standpoint cells can be dissected into particles such as chromosomes, microsomes, mitochondria, etc., each of which can be reduced to smaller particles, thence to molecules and finally to atoms and parts of atoms. But, from the standpoint of biology, where does life end in the process of reducing the building blocks of a life unit into smaller and smaller subunits?

To look at the problem in another way, consider the mechanism and results of cell division. A tissue cell divides and produces two daughter cells which if kept in the same environment maintain apparently identical phenotypes, and barring mutation, produce in the next generation four nearly identical cells. Obviously, to obtain truly identical cell products in mitosis, parts must be duplicated in order to duplicate the whole. It is known that the chromosomes are duplicated and that each daughter cell receives identical sets in mitosis. The precise duplication of these is important, since an error can cause grave disturbances in the phenotype, or even immediate death. Is an equally precise division of the cytoplasm of equivalent importance? The facts seem to be as follows: Cytoplasmic division can be quite unequal without an appreciable phenotypic change resulting in the offspring, but it cannot always be determined whether the unequal division of the cytoplasmic constituents is quantitatively or qualitatively unequal. What can be stated affirmatively, however, is that *some* cytoplasm seems always necessary. A nucleus alone is not a cell and gives even less evidence of life than an enucleated cell. It would appear therefore that the duplication of elements of both parts of the cell is necessary to provide materials to produce an entire cell. The nucleus, despite its undoubted importance, cannot make a complete cytoplasm, nor can the cytoplasm fashion a cell capable

of dividing and producing more cytoplasm generation after generation. The answer to the question concerning the smallest possible life unit would then be the cell, for this is the smallest unit capable of duplicating and producing living entities which can in turn do the same.

Although the cell may be considered the smallest unit capable of reproducing another living unit, it is not the smallest reproducible unit. Units such as genes maintain their biological integrity from generation to generation in a seemingly independent fashion from the rest of the cell. To emphasize the importance of these units in the maintenance of biological continuity they are sometimes described as being autocatalytic, self-duplicating or self-reproducing. These terms need to be examined closely as to precisely what they imply. All three are generally used as synonyms of one another, so to discuss one is to discuss the others. Autocatalysis is a term borrowed from chemistry. As used by the chemist it describes a type of reaction in which one of the products acts as a catalyst of the reaction. The product which so acts is termed an autocatalyst, since it directly catalyzes its own production. A curve showing change in rate with time of such a reaction is exponential, and if the substrate is not infinite in amount, becomes an S-shaped curve quite comparable to a population growth curve.

Since both biological growth and autocatalysis can be described with the same type of curve, it is reasonable to assume that the over-all kinetics of the two are similar. This does not, however, mean that every unit in the cell which maintains its integrity generation after generation is autocatalytic in the sense that it *directly* catalyzes its own production. Such units can well be parts of cyclic systems in which they may or may not exert a catalytic role. In the process of completing the cycle the unit is reproduced as an intermediate. It in turn may then act as a catalyst of other cyclic systems, products of which may catalyze its synthesis. It can be demonstrated mathematically that if the rate of synthesis of two cellular constituents is determined for each by the concentration of the other, then the increase in each will follow an autocatalytic or exponential curve (278). Thus if each part of the cell has a mutually catalytic effect upon the other, it is no wonder that each part should exhibit autocatalytic growth.

To take the example of an enzyme, which is increasing in amount exponentially (see increase in galactozymase, p. 307), it is seen that if its activity determines in part the metabolic activity of the cell which

is necessary for its synthesis then its increase will be autocatalytic in its kinetics. The enzyme is not truly an autocatalyst in itself—it is part of an autocatalytic system. Each of the members of the system has but one primary function, to catalyze some reaction—heterocatalysis. Its products in turn affect the synthesis of the catalysts indirectly—"pseudo" autocatalysis. Autocatalysis and heterocatalysis are therefore in this sense simply different aspects of the functioning of each catalyst or enzyme, each of which has but one function, catalysis.

Spiegelman (581) has maintained that the autocatalytic kinetics of galactozymase increase in activity in yeast adapting to galactose indicates that the galactozymase enzyme molecules duplicate themselves as cells would, generation after generation. Hence he separates the autocatalytic and heterocatalytic functions. Monod and Cohn (435) have pointed out that galactose is an energy source, and it would be expected that the energy derived from its breakdown would be in turn used in the synthesis of the enzyme or enzymes catalyzing the reaction. If an enzyme inductor is used which cannot be utilized as an energy source, and the other sources of available energy are limited, then it can be shown that the increase of the enzyme with time describes a curve which approaches being hyperbolic rather than exponential (435). Such a curve would be expected if the enzyme were formed through the action of other catalysts operating on substrates which are present in limiting concentrations, or more directly from preformed protein molecules which are induced to take on a new catalytic role as the result of actual contact with inductor molecules.

The Duplication of Genes

Current hypotheses concerning the duplication of genes are inclined to separate completely the heterocatalytic from the autocatalytic gene functions, and to postulate an origin for new genes based on the concept of antibody production by antigen as advanced by Pauling (477). The gene is pictured as a template which draws from the surrounding milieu the materials for the construction of a new gene which is effected as a one step process. This hypothesis has been advocated (161, 711) on the grounds that it is far simpler to conceive than one which requires each gene being built step by step through a series of reactions. The latter would require that all genes be occupied directly or indirectly in one another's synthesis, and therefore it might be expected to lead to extremely widespread mutation among many

genes after one of them has mutated. The fact that this has been detected in a very few instances (see, for examples, Chapter 3, p. 47) does not, however, argue either for or against either hypothesis, since too little is known of the mutation phenomenon. On the other hand, it is not necessary to assume that a gene loses all activity when it mutates. Even though a mutation causes a change in phenotype, it need not result in a change in other genes the synthesis of which is in part under the control of the mutated gene. It must also be recognized that there are always a great many mutations which result in complete lethality and hence cannot be analyzed for their specific effect in the cell. These "drastic" mutations may cause, among other effects, secondary mutations of the type mentioned above.

Among the very few facts known about the synthesis of genes, two may be set down as being of greatest significance. (1) A gene can, as far as is known, be synthesized only in the presence of another like it. Like cells, genes come only from preexisting genes. Furthermore genes are duplicated as parts of units, the chromosomes. A normal chromosome present in the same cell with a deficient homologue cannot make up the deficiency of its incomplete partner. (2) The synthesis of a second gene in the presence of its "model" is a very precise process. Few "mistakes" are made which are detectable as mutations affecting the phenotype. On the other hand, a mistake once made resulting in a detectable mutation can be duplicated as such indefinitely.

That the template hypothesis is capable of providing a rational explanation for both of these facts is obvious. If the hypothesis that each gene is part of a cycle of reactions in which it acts as an intermediate is invoked (109) (see p. 314), it is necessary to assume that it is an indispensable part of the cycle. Without it the cycle cannot operate, and the gene cannot be duplicated. If the gene changes, the cycle changes, and the duplicate gene produced as a result is like the mutated gene. The interesting observation that a normal chromosome cannot rectify a deficiency in its homologue in a cell heterozygous for a deficiency, is difficult to explain by either hypothesis.

It should be emphasized that neither one of the two hypotheses discussed—the contact template versus involvement in a cycle—is simple. It is as difficult to visualize the forces which must be postulated to be at work to draw the basic building blocks of a future gene to its model, as it is to visualize the complex series of interlocking cycles necessary to account for gene synthesis by many steps.

The two hypotheses have been presented as alternatives to emphasize their differences, but they are not necessarily mutually exclusive or antithetical. A combination of both is tenable. What is important is to recognize that it is not necessary for the gene's heterocatalytic function to be completely divorced from its autocatalytic function. Neither hypothesis is self-sufficient, nor are they so when combined. Too many factors exist which cannot be explained by any hypothesis now current.

The Duplication of Cytoplasmic Constituents

The problems that arise in a consideration of the duplication of chromosomes are also germane in connection with the remainder of the cell. Unfortunately, even less is known about the duplication of the extragenic elements of the cell than is known about chromosome duplication, but this has not hindered speculation concerning it. As will be made clear in the following section, there is adequate evidence to prove the existence of genetic continuity of elements of the cytoplasm, but the association of this continuity with specific particles in the cytoplasm has been found possible in only a very few cases.

Centrioles, mitochondria, plastids, microsomes, and Golgi bodies are among the microscopically visible cytoplasmic particles which undoubtedly have more than a storage function and which conceivably may have genetic continuity in all organisms in which they occur. Centrioles are universally present in animal cells and in the cells of lower plants. In many animals they can be seen to divide and duplicate, hence maintaining a continuity of relationship from one generation to the next (661, 694). There is considerable doubt, however, that this continuity is genetic in all organisms, since centrioles may apparently arise *de novo* by treatment with external agents, as in the sea urchin (694), or perhaps even from the nucleus, as in certain of the lower plants (719). Lwoff (386), like most protozoologists, places great importance on the centrioles or kinetosomes of the ciliate protozoa which can be clearly demonstrated to have a continuity from generation to generation. He has endowed them with a genetic significance comparable to that of genes, and developed an interesting and illuminating theory regarding their role in development.

Mitochondria are undoubtedly essential constituents in animal cells. Their metabolic significance has already been discussed at length in the preceding chapters. In plant cells they are presumably of equal

significance (see Newcomer (454, 455) for review). Despite, however, their undoubted significance and the indications of continuity through successive generations because of their almost universal occurrence in all cells of all organisms, it is possible that they may arise *de novo* from invisible cytoplasmic components not having the characteristics of mitochondria. Universal occurrence is no proof of physical and genetic continuity such as is displayed by genes.

The occurrence of physical continuity of mitochondria through the male gametes is amply demonstrated in the fascinating observations of Wilson (695) on several species of scorpions. In the centrurid scorpions (whip scorpions) the mitochondria form a closed ring at the onset of spermatogenesis which appears in the primary spermatocytes just outside the spindle and generally parallel to it. In the first metaphase the ring is elongated in a plane parallel to the long axis of the spindle. The ellipse so formed then breaks at the ends, producing two mitochondrial rods lying parallel. Each of these then breaks transversely and equally at telophase, giving each secondary spermatocyte two mitochondria. In the second division an equal transverse division again occurs, and therefore four spermatids each with two mitochondria are formed.

The spermatogonial mitotic divisions unfortunately could not be followed as easily as the meiotic divisions, but it is clear, nonetheless, that the mitochondria form the ring only in spermatogenesis. During mitotic divisions the mitochondria show a bipolar segregation so that each daughter cell receives a share of them. The origin of new mitochondria during the course of continued mitosis is, however, not understood, though presumably it may be by transverse division as in spermatogenesis.

In the non-centrurid scorpions, the ring is not formed during spermatogenesis, but an equivalent equal distribution of mitochondria is nonetheless attained. For example, the scorpion, *Opisthacanthus,* shows 24 mitochondria in its primary spermatocytes. These are generally divided equally in the first division and again in the second division so that 4 spermatids each with 6 mitochondria are regularly formed from each primary spermatocyte. Out of about 500 cases examined by Wilson, 76% demonstrated 4 spermatids with 6 mitochondria, 17% with 5, and 7% with 7. Wilson (695) interpreted these data as evidence for a mechanism insuring an approximately equal distribution, but he did not presume that anything more than a random assortment is indicated. Although non-disjunction of chromosomes during meiosis occurs to a much lesser degree than is ex-

hibited here by unequal distribution of mitochondria in scorpions, it is possible that Wilson tended to belittle the significance of his own observations.

Since the mitochondria distributed to each spermatid are incorporated into the cytoplasm of the mature spermatozoa, and are therefore presumably passed on to the next generation, it is quite possible that mitochondria do have a genetic continuity in the scorpions. Similar observations on the regularity of mitochondrial distribution during meiosis have been made in a number of insects (487) and in the protozoan *Spirostomum* (177). The phenomenon may be somewhat more widespread than heretofore thought. Perhaps better methods of demonstrating mitochondria in dividing cells will make it possible to study this important mechanism to better advantage in the future and to determine whether it is a general phenomenon or merely a natural oddity occurring in certain arthropods and Protozoa.

The evidence for the continuity of chloroplastids in the green plants is somewhat better than that for mitochondria. But even here the crucial observations needed for proof of genetic continuity are wanting. In the lower green plants such as the algae, the chloroplastids can be seen to divide and the division products distributed among the daughter cells resulting from mitosis. In certain of the Chrysophyceae such as *Rhizochrystis* and *Myxochrysis* the chloroplasts do not always divide as rapidly as the remainder of the cell, in which event cells are produced without plastids (471). This loss of chloroplasts during cell division may account for the fact that many algal groups have colorless counterparts which are identical in appearance to the green forms, and differ only in the absence of chlorophyll and hence their mode of nutrition. The loss of chloroplasts might also be caused, of course, by gene mutations, as has been amply demonstrated in the higher plants (p. 329).

The disappearance of chlorophyll and chloroplastids has been studied experimentally in various species of *Euglena*. Most *Euglena* species and indeed many other algae which are not obligate autotrophs lose their chlorophyll pigment when cultured in the dark but quickly regain it when returned to the light. *Euglena gracilis* has been shown by Baker (16) to lose all signs of chloroplastid structure with the disappearance of chlorophyll. The chloroplastids might therefore be considered to arise *de novo* in colorless individuals in the presence of light. On the other hand, Lwoff and Dusi (387) working with *Euglena mesnili* found that the complete loss of chloroplastids in this species in the course of dark growth resulted in colorless strains which

were completely incapable of producing chlorophyll or chloroplasts on being returned to the light. Furthermore, the treatment of *Euglena gracilis* with sublethal doses of streptomycin rids the cells of their chloroplasts even in the light. The colorless strains so obtained are completely incapable of regenerating chloroplasts (497). None of these observations affords conclusive proof for the genetic continuity of chloroplastids as such, nor provides evidence against it. It is obviously extremely difficult to determine whether a particle can be duplicated only in the presence of a preexisting particle unless genetic tests are possible.

In the flowering plants chloroplastids arise from colorless proplastids which may be genetically related to mitochondria (375), but no direct experimental proof of this is at hand (see review of Newcomer (454, 455).) Evidence that chloroplastids in this group have continuity through successive generations is discussed in detail in the next section.

The remaining important formed elements, the microsomes and Golgi bodies have, like the particles discussed above, been accused of continuity by some authors and of *de novo* origin by others. There is no particular evidence worth quoting to support either view. That they are duplicated is certain, for Golgi bodies are invariable constituents of all animal cells, and microsomes (or chromidia) are presumably universally distributed, although the best evidence for their existence comes from work on animal tissue cells.

The Establishment of Proof That a Particle or Subunit Has Genetic Continuity

For a cell unit to have a role in the continuity of organization of the cell, it must (1) determine some characteristic of the cell, (2) be duplicatable, and (3) be distributed to the daughter cells during cell division. These might be called the minimum requirements for genetic continuity. To demonstrate that a cell unit fulfills these requirements, it is first necessary for it to be recognized by a mutation that causes a phenotypic change. Following this, it must be shown that both the mutant and the normal condition can be maintained indefinitely through successive cell generations. If such continuity is demonstrated, the existence of something inheritable that is a determinant is demonstrated, but the determinant is not localized in any part of the cell.

In an organism capable of sexual reproduction a cross between individuals carrying contrasting phenotypes produced by the mutation

will demonstrate, by the ratios obtained in the offspring, whether the determinant is part of the chromosome structure of the cell. If the ratios are non-Mendelian, and if the mutant phenotype is inherited uniformly by the offspring when the mother is mutant in phenotype, but not at all in the reciprocal cross when the father is mutant, then genetic continuity through the cytoplasm rather than the nucleus is indicated. The assumption made in arriving at this conclusion is that the male gamete contributes a nucleus, but no cytoplasm. Actually this cannot be definitely proved in any known example. Nearly all, if not all, animal sperm contains cytoplasmic constituents, and it seems quite certain that the pollen tube of the flowering plants may in some species introduce male cytoplasm to the egg along with the sperm nucleus. However, if the mutant characteristic persists in being transmitted only through the sex which presumably contributes the largest part, or all, of the cytoplasm then it is justifiable to conclude that its continuity is through the cytoplasm. The possible role of the chromosomes of the female in maintaining the difference can be eliminated with a high degree of probability by successive back crosses to the normal male strain which should effectively replace the original set in the female line.

Once it is established that a characteristic is determined by either a specific condition in the cytoplasm or the nucleus, it is next generally desirable to localize the determinant more precisely. It is a relatively simple matter to locate a determinant on a specific chromosome, if other genes are available as markers. On the other hand, it is not so simple to locate a determinant in the cytoplasm with visible cytoplasmic particles.

If a particular characteristic is always associated with a specific type of particle, chlorophyll production with chloroplasts for example, and the particle once absent is not reconstituted in colorless cells, then it is usually assumed that the particle is the inherited determinant which can arise only from preexisting ones like itself. Such a conclusion, however, does not reckon with the possibility of the non-particulate part of the cytoplasm being the determinant. It need only be assumed that this portion of the cytoplasm changes to produce metabolic conditions in which chloroplastids cannot develop. If two types of visibly different particles can coexist in the same cell, this latter objection can be eliminated, provided it can be also shown that in introducing one of the particle types only the particle and no other cytoplasmic components from the donor is transmitted.

Technically, this is impossible to prove when the particle is introduced in the normal process of fertilization (or of hyphal fusion among the fungi). Actually conclusive proof can only be obtained by introducing washed particles artificially. Up to the present this feat, which is well within the realm of possibility as demonstrated by successful infection of cells with pure strains of virus, has not been accomplished with normal cellular constituents.

A second possible method is to use the geneticist's chief tool for the dissection of the genotype, recombination determinations. To utilize this method it would be necessary to have within a single cell two different types of particles which interact to produce a third and fourth type in equal numbers. A segregation of the four types and further recombinations between unlike ones would enable the observer to identify the characteristic with the particles. This has proved feasible with phage (273) and the possibility exists that it may be feasible with mitochondria, chloroplasts, etc., provided that enough cytoplasmic markers are first identified. On the other hand, if the cytoplasmic particles consist of single inherited entities only, this method cannot be employed.

2. Extrachromosomal Inheritance

It is a well-known fact that the phenotype of a cell having a given genome can be modified extensively by changes in environmental conditions. This is a reversible and continuous process with its origin in the flexibility of a very complex pattern of interacting and thus interdependent biochemical reaction series and cycles. The limits of, and the specific characteristics that arise as a result of, this flexibility are different for each gene complement even though these differences may be small and go undetected. The inherited phenotype includes this reversible elasticity of the systems, and its expression in successive generations of genetically homogeneous progeny is expected to be influenced in the same way by environmental changes. It is apparent, however, that within the limits of possible expressions of phenotypes with a given genome there are stable states that show self-limiting flexibilities. These properties of the system may be inherited cytoplasmically. As already discussed they have frequently been correlated with observable changes in cytoplasmic particles and attributed to mutation of hypothetical cytoplasmic units of inheritance. Among the various names that have been applied to describe these real or hypothetical units are pangenes, bioblasts, plasmagenes,

chondriogenes, cytogenes, provirus, genoids, plasmids, protomeres, and plastogenes. None of these terms is used here since they are not necessary to describe the phenomena of cytoplasmic inheritance. The mechanism of transmission of cytoplasmic characters is not well enough understood to permit an analysis.

The Demonstration of Cytoplasmic Inheritance

It is not possible here to review the large number of examples of non-chromosomal inheritance that have been described since the first discovery of the phenomenon in 1909 (24,117). Most of the cases have been found in higher plants or microorganisms with relatively few in animals. These are discussed here under the following arbitrary and certainly not mutually exclusive categories:

1. Examples in which cytoplasms and genomes were established through interspecific crosses.
2. Examples in which different cytoplasms were established within the same species without known direct chromosomal influence.
3. Examples in which cytoplasmic characters can be related to the result of a particular chromosomal mutation.

A very significant problem in the study of cytoplasmic inheritance is that concerned with devising means of recognizing the phenomenon when it occurs. One of the earliest developed means of doing this was devised from investigations on crosses between related species of higher plants. The most extensive and informative work of this kind that has been done is that of Michaelis (410) and collaborators on various species of the willow herb, *Epilobium*. This is a small flowering plant of the family *Onagraceae* which is normally diploid, develops air-borne seeds, and has pollen grains that produce fertilization without transfer of any large amount of cytoplasm from the paternal parent. This last point is particularly important since it permits a measure of inheritance based almost entirely on the properties of the mother cell when the gene complements of the two parents are alike. In an exemplary experiment *Epilobium luteum* was crossed to *Epilobium hirsutum* through 25 generations over a period of 23 years through the following scheme:

$$[(luteum\ ♀ \times hirsutum\ ♂)\ ♀ \times hirsutum\ ♂]\ ♀ \times hirsutum\ ♂$$
for 25 generations (strain lh^{25})

In this way the genome of *hirsutum* was gradually introduced into the mother cell of *luteum* to provide a strain with the total cell characteris-

tics very similar to *luteum* but with gene constitution of *hirsutum*. Reciprocal crosses were then made as follows:

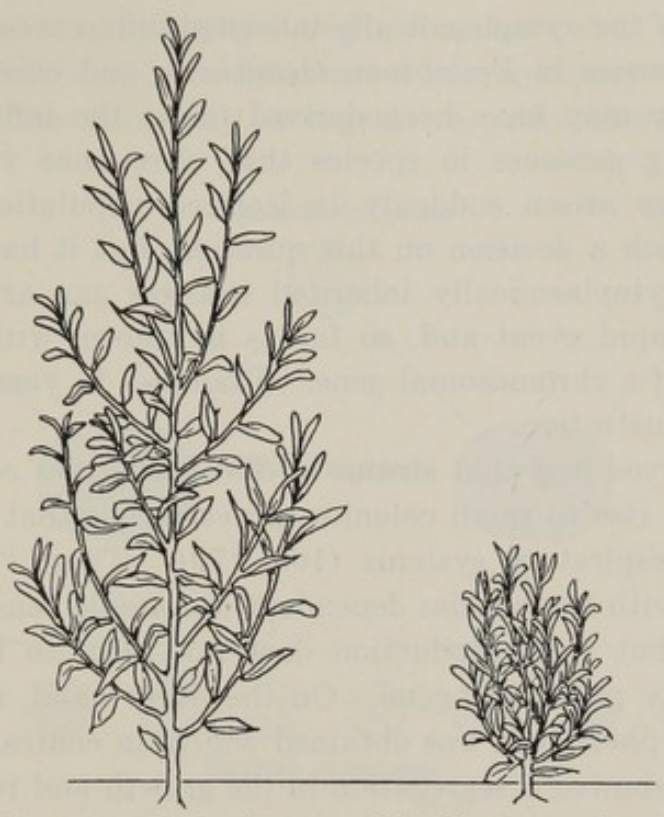
(1) lh^{25} ♀ × *hirsutum* ♂

(2) *hirsutum* ♀ × lh^{25} ♂

The male and female parents in both of these crosses now carry essentially the same genome but they differ by whatever contribution is made by the cytoplasm of the maternal parent. The phenotypic expressions of these cytoplasmic differences were then evaluated by comparisons of the progeny from the reciprocal crosses.

Examinations of progeny of a large number of different lines that were developed as described above yielded incontestable evidence for cytoplasmic inheritance with respect to a great variety of phenotypic characteristics. These include differences with respect to lethality, sterility, plant anatomy, flower color, heterosis, cytoplasm viscosity, permeability, sensitivity to poisons, fungus resistance, reaction to temperature and light, enzyme activities and concentrations of metabolites. The variety of changes observed here thus encompasses nearly the same phenotypic expressions that can be observed as the result of mutation of nuclear genes. The differences observed vary in degree among the progeny from one cross, and the ranges of expression sometimes overlap considerably among the progeny from reciprocal crosses, but again this is also true of characters altered by chromosomal mutation although in the latter case we usually select non-overlapping characters for study. Some examples of variations in size and character of plants derived from reciprocal crosses among different races of *Epilobium* are shown in Fig. 92.

Interspecies crosses in *Oenothera* have provided further good examples of the cytoplasmic inheritance in higher plants (511). Here reciprocal crosses can be examined directly since segregations of chromosomes at meiosis is not random as in *Epilobium*. They are associated in complexes that move as units in the first anaphase division. *Oenothera odorata* carries the two chromosome complexes *v* and *I* whereas *Oenothera Berteriana* carries the two complexes *B* and *l*. Each complex can be identified cytologically, and crosses between these two species give rise to the new combinations *B v*, *B I*, *l v*, and *l I*. Results from reciprocal crosses describing the effects of the maternal cytoplasm on plants carrying these new combinations of chromosome complexes are summarized in Table 46. As shown, the hybrid products of these crosses have growth and viability charac-



Jena ♀ × München ♂ München ♀ × Jena ♂

Fig. 92. Results of reciprocal crosses between closely related species of *Epilobium,* showing cytoplasmic inheritance of plant size. After Michaelis (409*a*).

teristics that are as dependent on which species served as the maternal parent as on the genome. Subsequent selfing of a viable hybrid usually gives rise to plants with the same characteristics as the hybrid, and after several generations a back cross to restore the normal chromosome complexes will give normal plants. Slow and heritable changes have been observed on continued self-crossing of one of the hybrids.

Table 46. Cytoplasmic Inheritance Shown by Reciprocal Crosses in *Oenothera* *

	Cross: ♀ *O. Berteriana* × ♂ *O. odorata*		Cross: ♀ *O. odorata* × ♂ *O. Berteriana*	
	B l	*v I*	*v I*	*B l*
Chromosome Complex	Appearance of Hybrid		Appearance of Hybrid	
B v	Non-viable		Normal	
B I	Normal		Weak and yellow	
l v	Mostly normal, lower leaves sometimes yellow		Weak and yellow	
l I	Mostly normal, lower leaves sometimes yellow		Non-viable	

* From a review by Weier and Stocking (680).

The origin of the cytoplasmically inherited differences found through interspecific crosses in *Epilobium, Oenothera,* and other organisms is obscure. They may have been derived under the influence of gradually diverging genomes in species that were once very similar or they may have arisen suddenly in isolated populations. It is not possible to reach a decision on this question, but it has been demonstrated that cytoplasmically inherited changes can arise within one species as a rapid event and, so far as is known, without induction by mutation of a chromosomal gene. Examples in yeast and in *Neurospora* are illustrative.

It was observed first that strains of *Saccharomyces cerevisiae* spontaneously give rise to small colonies of yeast cells that have deficiencies in their respiratory systems (169, 170). These "vegetative petites" appear with frequencies dependent on genetic constitution of the mother cells, but their production does not seem to be conditioned directly by any particular gene. On the other hand, a mutant with a very similar phenotype was obtained which, in contrast to the vegetative petites, showed a segregation in the growth and respiratory system characteristics as though the phenotype is due to a single gene mutation. Results of crosses involving normal yeast, vegetative petite, and segregational petite are shown in Fig. 93. As indicated diagrammatically by the solid centers the genome of the normal strain and the vegetative petite are considered to be the same, and each of these gives identical diploid zygotes and ascus products in most crosses. Crosses of normal to vegetative petite gives normal zygotes and normal ascospores. These crosses are self-consistent and show that the phenotype of the vegetative petite is lost when these cells fuse with cells that carry a normal gene complement. Unfortunately the vegetative petites do not yield diploid zygotes which produce ascospores among themselves so the possible interactions of two of these cells cannot be evaluated. Still, the evidence for cytoplasmic inheritance is convincing for this example, and it is supported further by analogy with a somewhat similar case in *Neurospora* (426, 429).

In this organism an analysis of cytoplasmic inheritance of the type described for *Epilobium* can be carried out. That is, even though either mating type of the mold can be used as the maternal parent, the fertilizing parent apparently contributes little or no cytoplasm to the zygote cell, and thus reciprocal crosses can be made (see Fig. 28, p. 100). In addition the strains of mold that have been investigated are fertile in all combinations of intercrosses. The strains of *Neuro-*

spora used were of spontaneous origin, they have slow growth characteristics, and they also have deficiencies in respiratory systems though not in exactly the same way as vegetative petite (see p. 142 for dis-

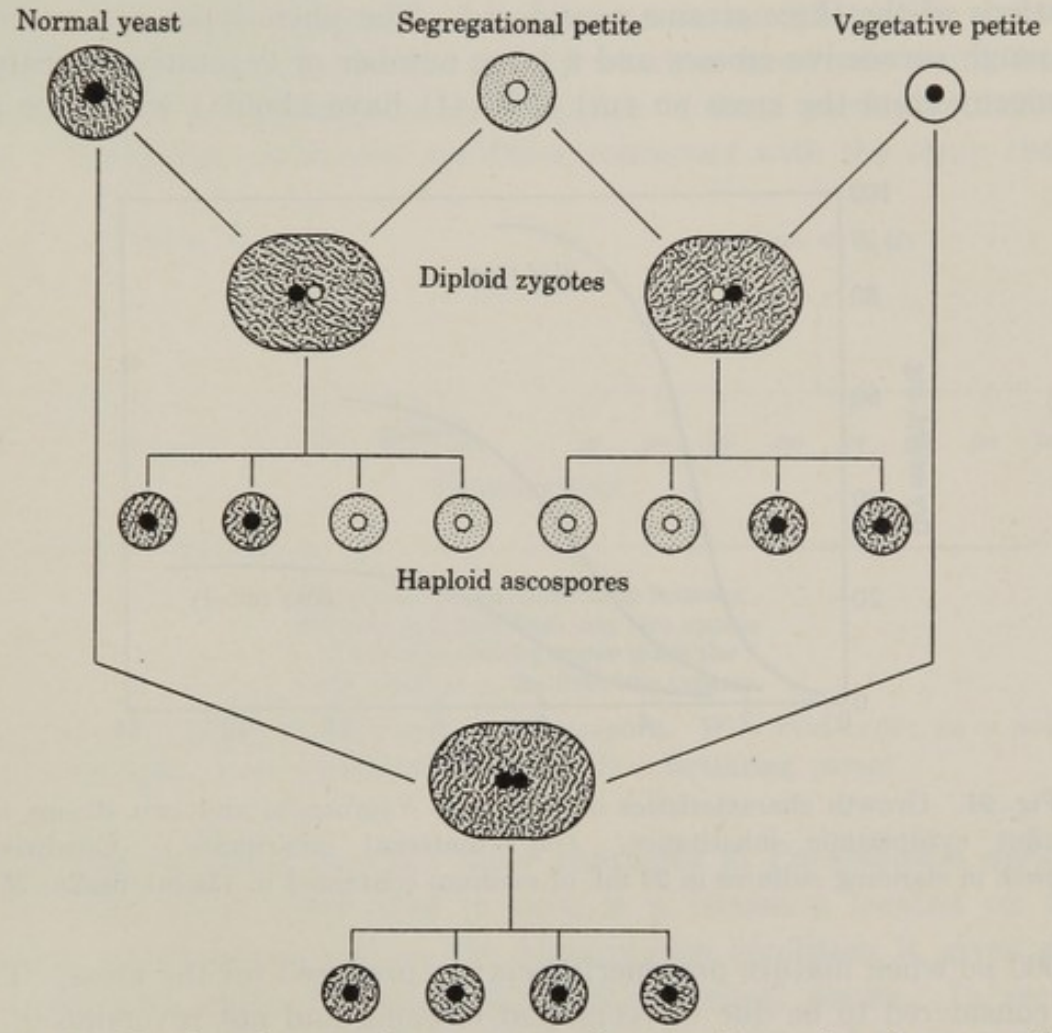


Fig. 93. Results of crosses between normal and petite strains of yeast. After Ephrussi (169). The solid center dots indicate a normal complement of nuclear genes; the open circles indicate a mutant nuclear gene. Abnormal cytoplasms are indicated by the light dotted or clear area around the nuclei. The nuclei would actually be fused in the zygotes but would contain a normal and a mutant gene for the segregational and petite character. In this organism whole cells fuse to form zygotes, and so both parents contribute cytoplasm as well as nuclei.

cussion of respiratory systems). Growth characteristics of wild type and two strains that exhibit cytoplasmic inheritance are shown in Fig. 94. Any one of these strains, when allowed to grow on the appropriate medium, will cover the agar and produce large numbers of mature protoperithecia that are receptive to fertilization by conidia of the opposite mating type from any one of the other strains or from

itself. The progeny from each cross made in this way, all or very nearly all, have the phenotype of the protoperithecial parent. Illustrative examples are given in Fig. 95.

Similar results were obtained with successive crosses in all combinations of the three strains considered. The phenotypes are retained through successive crosses and a large number of vegetative transfers. Progeny from the cross *po* (m) × *W* (f) have about 1 wild type per

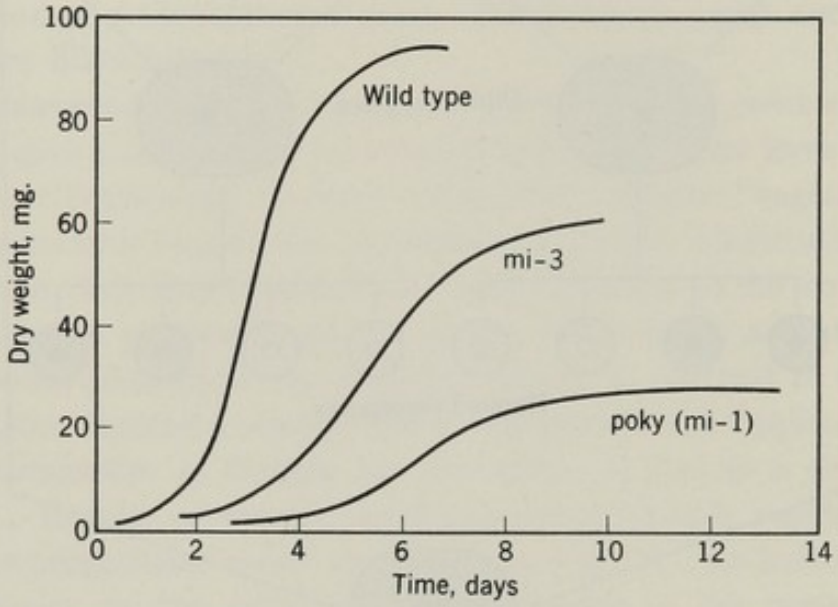


Fig. 94. Growth characteristics of wild-type *Neurospora* and two strains that exhibit cytoplasmic inheritance. (*mi* = maternal inheritance.) Conditions: growth in standing cultures in 20 ml. of medium contained in 125-ml. flasks; 25°C.

1,000 *po* when mature protoperithecia are prepared for the cross. This is considered to be due to reciprocal crossing and not reversion of *po* since crosses of *po* × *po* have not yielded any wild types among more than 20,000 progeny. Growth rates of different isolates of *po* and *mi*-3 vary about ±15%, as do those of normal wild-type strains, but the phenotypes on a growth basis do not overlap and the phenotypes as defined on a biochemical basis (p. 339) also remain within definite limits. These strains of *Neurospora* therefore provide clear and well-defined examples of non-Mendelian inheritance.

As discussed further on p. 187 two nuclear gene mutants of *Neurospora* (C115 and C117) that have slow growth characteristics and aberrations in their respiratory systems have also been obtained. These show a normal segregation of 1:1 when crossed to wild type, but neither will serve as protoperithecial parent so the reciprocal crosses have not been made successfully. However, the double mu-

tant of C115 with a certain suppressor will serve as protoperithecial parent, and segregations among the progeny from such a cross are normal for this combination. Thus it is probable that cytoplasmic inheritance is not involved with strain C115 at least.

The third class of cytoplasmic inheritance as arbitrarily defined here for purposes of discussion is that which is known to result from or be directly conditioned by the mutation of some particular nuclear gene. Examples considered are those concerned with the *iojap* char-

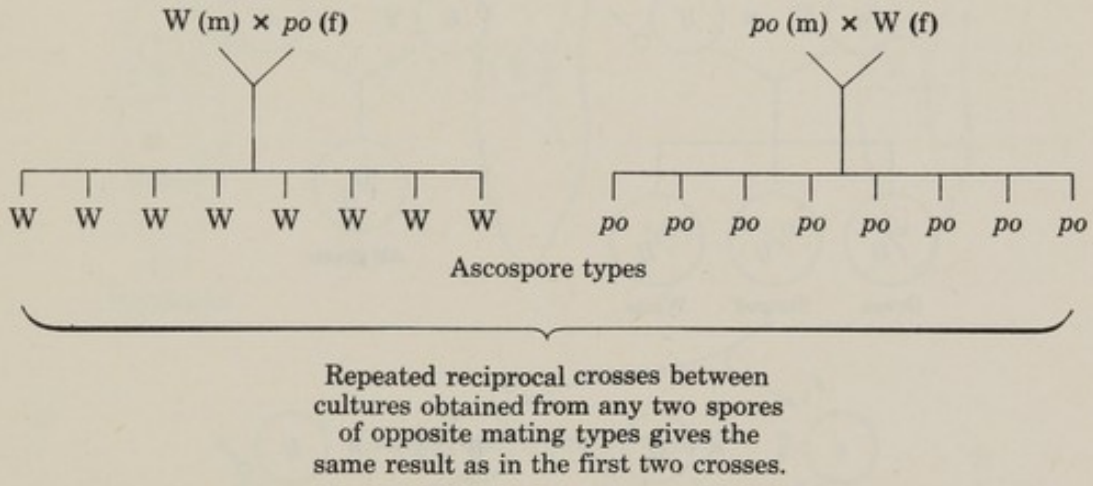


Fig. 95. Cytoplasmic inheritance in *Neurospora*. *W* = wild type; *po* = *poky*; (m) = maternal or protoperithecial parent; (f) = fertilizing parent.

acter in maize (514) and the *killer* character in *Paramecium aurelia* (573, 576). The *iojap* gene in corn is a recessive located on the seventh chromosome, and in the homozygous condition it gives rise to *green* and *white* or occasionally *yellow* striped plants. In reciprocal crosses with a normal strain it yields a variety of progeny when *iojap* (*ij*) is used as the maternal parent but only *green* plants when the maternal parent is normal (*Ij*). These results are diagrammed in Fig. 96 which also includes a summary of the results of crosses from F_1 *striped* plants. All F_1 plants from the first reciprocal crosses have the same constitution with respect to chromosomal genes as shown in the figure. The *white* F_1 seedlings do not mature, but the *striped* seedlings do and can be used in further crosses. As shown they give rise to either *ij* or *Ij* mother cells, and when these are fertilized with normal pollen both types produce green, striped, and white plants. Thus, it is established that, although the recessive *ij* gene is necessary to establish the inherited cytoplasmic condition, this state will continue after replacing the gene with its dominant allele.

It is of course possible that established cases of cytoplasmic inheritance may have arisen in this fashion.

Cytoplasmic inheritance in *Paramecium aurelia* has been investigated very extensively with respect to the killer character, ciliary antigens and mating types (576). These are of special interest because they, along with the character of CO_2 sensitivity in *Drosophila*

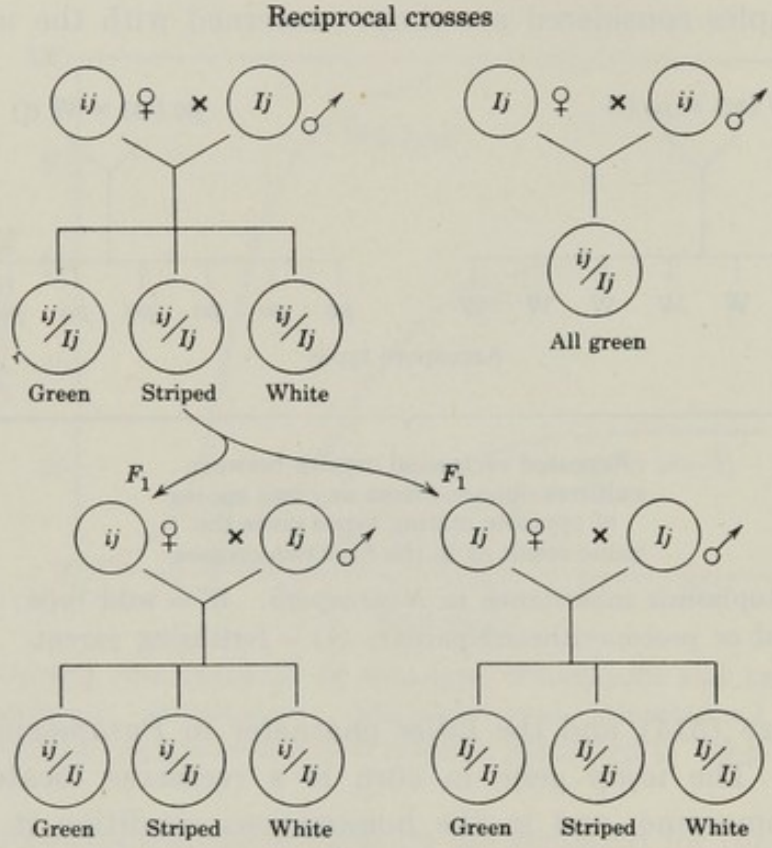


Fig. 96. Cytoplasmic inheritance in maize, showing the influence of the recessive gene, *ij*. *ij/ij* gives green and white striped plants, and some with yellow stripes.

(376), represent the only well-established examples of cytoplasmic inheritance in animals. Inheritance of antigenic specificity is considered in connection with environmental influences (p. 295), and only inheritance of the killer character will be discussed in detail in this section.

The exchange of nuclear genes in *Paramecium* takes place by a partial fusion of two animals to form a small cytoplasmic bridge between them. The nuclei from the two parents fuse by migration through this bridge, and after subsequent divisions and equal distribution of the new nuclei formed the bridge is sealed off and the two animals separate. They then reproduce by direct fission. An

important aspect of the conjugation process so far as cytoplasmic inheritance is concerned is the fact that the cytoplasmic bridge sometimes persists for a longer time than necessary for nuclear exchanges and permits an exchange of relatively large amounts of cytoplasm.

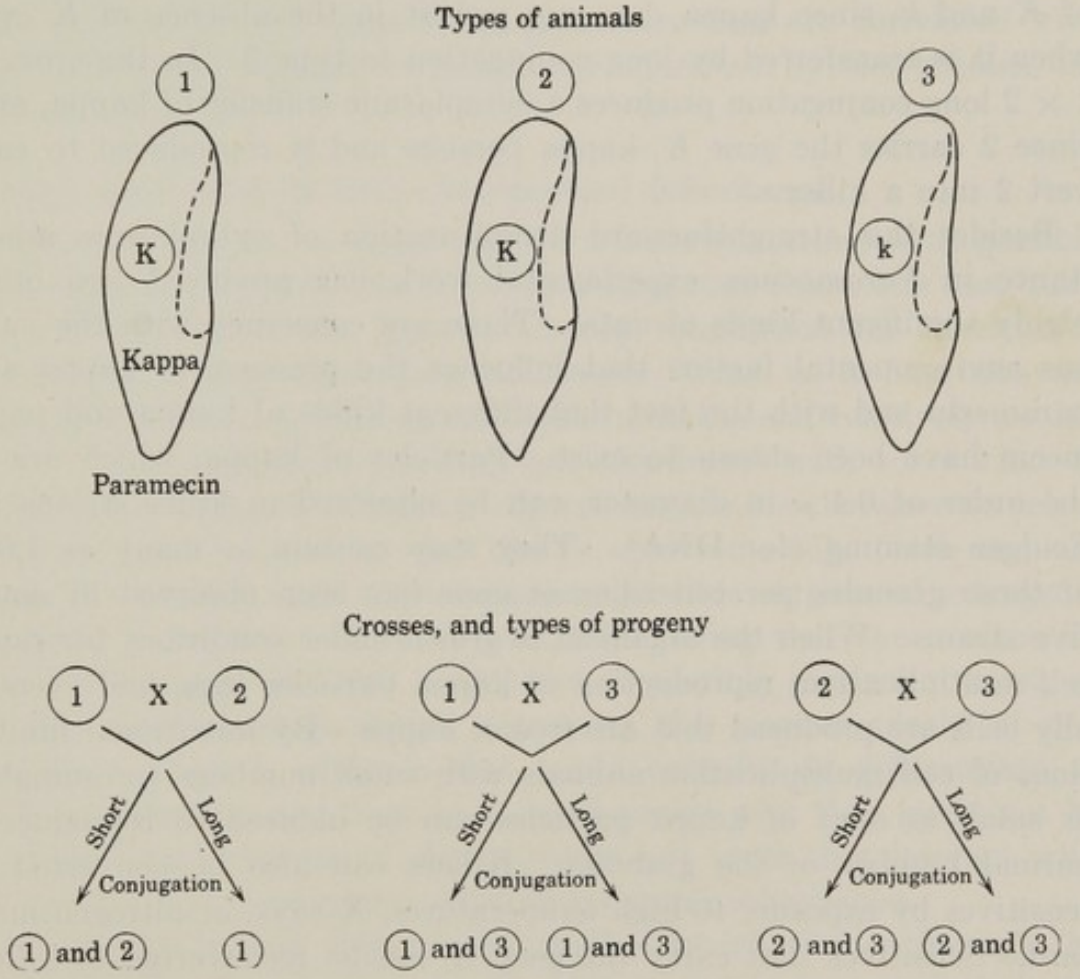


Fig. 97. Inheritance of the killer character in *Paramecium aurelia*. K = dominant gene; k = recessive gene; *kappa* = cytoplasmic unit present with K; paramecin = toxic substance produced in the presence of K and *kappa*. After Sonneborn (573, 575).

As shown in Fig. 97, cytoplasmic inheritance in *Paramecium* is clearly demonstrated on this basis by crosses and cytoplasm exchange among the three races indicated. Race 1, the killer strain, carries the dominant gene K in the nucleus, it produces large cytoplasmic particles *kappa*, and it releases other large particles called paramecin into the culture fluid. Both kappa and paramecin appear to contain large quantities of desoxynucleoprotein, and paramecin is only produced when kappa is present in the cells. Cells which do not contain kappa are sensitives and are killed by coming in contact with

paramecin produced by a killer. The sensitives are of two types (2 and 3 in Fig. 97), one of which carries the dominant gene *K* whereas the other carries only its recessive allele *k*. Killing does not occur during conjugation, and the results of crosses among 1, 2, and 3 are shown in Fig. 97. Crosses of 1×3 and 2×3 show only segregation of *K* and *k*, since kappa does not persist in the absence of *K* even when it is transferred by long conjugation to type 3. In the cross of 1×2 long conjugation produces a cytoplasmic transfer of kappa, and, since 2 carries the gene *K*, kappa persists and is reproduced to convert 2 into a killer.

Besides this straightforward demonstration of cytoplasmic inheritance in *Paramecium* experimental work has produced two other highly significant kinds of data. These are concerned with the various environmental factors that influence the presence of kappa and paramecin and with the fact that different kinds of kappa and paramecin have been shown to exist. Particles of kappa, which are in the order of 0.4 μ in diameter, can be observed in killer strains by Feulgen staining (for DNA). They may contain as many as 1,600 of these granules per cell whereas none has been observed in sensitive strains. When the organism is grown under conditions for rapid cell multiplication, reproduction of kappa particles lags, and eventually cells are produced that are free of kappa. By nutritional limitations of cell multiplication animals with small numbers (presumably as small as one) of kappa particles can be induced to reproduce a normal number of the granules. Killers can also be converted to sensitives by exposure to high temperatures, X-rays, or nitrogen mustard. Sensitives that carry the gene *K* can be reconverted to killers by the cytoplasmic transfer already described or by exposing them to a concentrated, cell-free suspension of ground-up killer animals.

That kappa and paramecin can exist in a number of forms with different biological specificities but still conditioned by the same gene *K* has been demonstrated (142). These different kinds of units were obtained by experimentally reducing the number of kappa particles per cell to a very low level and then permitting a regeneration period. Five types were obtained, distinguishable on the basis of the quantity or quality of paramecin produced. For example, an original strain of killer produces paramecin that causes blisters and cellular distortions of sensitives with death occurring in about 24 hours. Of two new cultures that were derived from this by the kappa dilution and regeneration technique, one produces a limited amount of

paramecin with killing characteristics like the original whereas the other produces a paramecin that causes sensitive animals to spin rapidly about the longitudinal axis and to die in about 8 hours. Other killer strains were obtained that produce paramecin that will kill different killers, although in no case does paramecin act on the animals that produce it. All these killer characters that are correlated with the presence or absence of kappa are inherited by the process described (Fig. 97).

On the Mechanism of Extrachromosomal Inheritance

The development of ideas and hypotheses concerned with possible mechanisms of transmission of extrachromosomal characters has, quite naturally, followed the pattern already established for inheritance of chromosomally determined characters. That is, it has been assumed that the cytoplasm must contain mutable and relatively autonomous organized structures that are distributed with some kind of regularity during cell divisions. It was reasonable therefore to select the cytoplasmic inclusions that can be observed easily (such as plastids, mitochondria, and microsomes) as possible carriers of extragenic heritable characters. It is somewhat surprising that the nucleus itself has not been included in this list of possible carriers since it, of all the cytoplasmic particles, segregates the most regularly at cell division. There is evidence that nuclei actually do differentiate as cells differentiate, and there is no good reason why they could not be simultaneously carriers of chromosomes and units that interact with the cytoplasm to determine extrachromosomal inheritance.

In connection with this general problem of inheritance through the action of the cytoplasmic constituents, it should be noted that one of the limitations in the detection of cytoplasmic inheritance lies in the fact that in most organisms the fertilizing parent supplies some cytoplasmic constituents along with a nucleus. This is usually much smaller in quantity than that of the maternal parent, but it cannot be ignored. In the examples of reciprocal crosses given here either the paternal parents supply an insignificant quantity of cytoplasm or the characters are so dominant that a little paternal cytoplasm is of no consequence. That dominance of this kind does exist is shown by the results of crosses in yeast and *Paramecium* where direct mixing of cytoplasms of the whole conjugating cells can take place. Thus, mixing cytoplasms of petite vegetative and normal yeast gives normal zygotes and segregants whereas mixing of cytoplasms of killer and *K* sensitives gives all killer progeny. On the other hand the killer phe-

notype is not dominant in the presence of the gene *k*. In *Oenothera* it has been shown cytologically that in some species pollen carries an appreciable contribution of plastids (and they presumably also carry appreciable amounts of other cytoplasmic constituents) into the

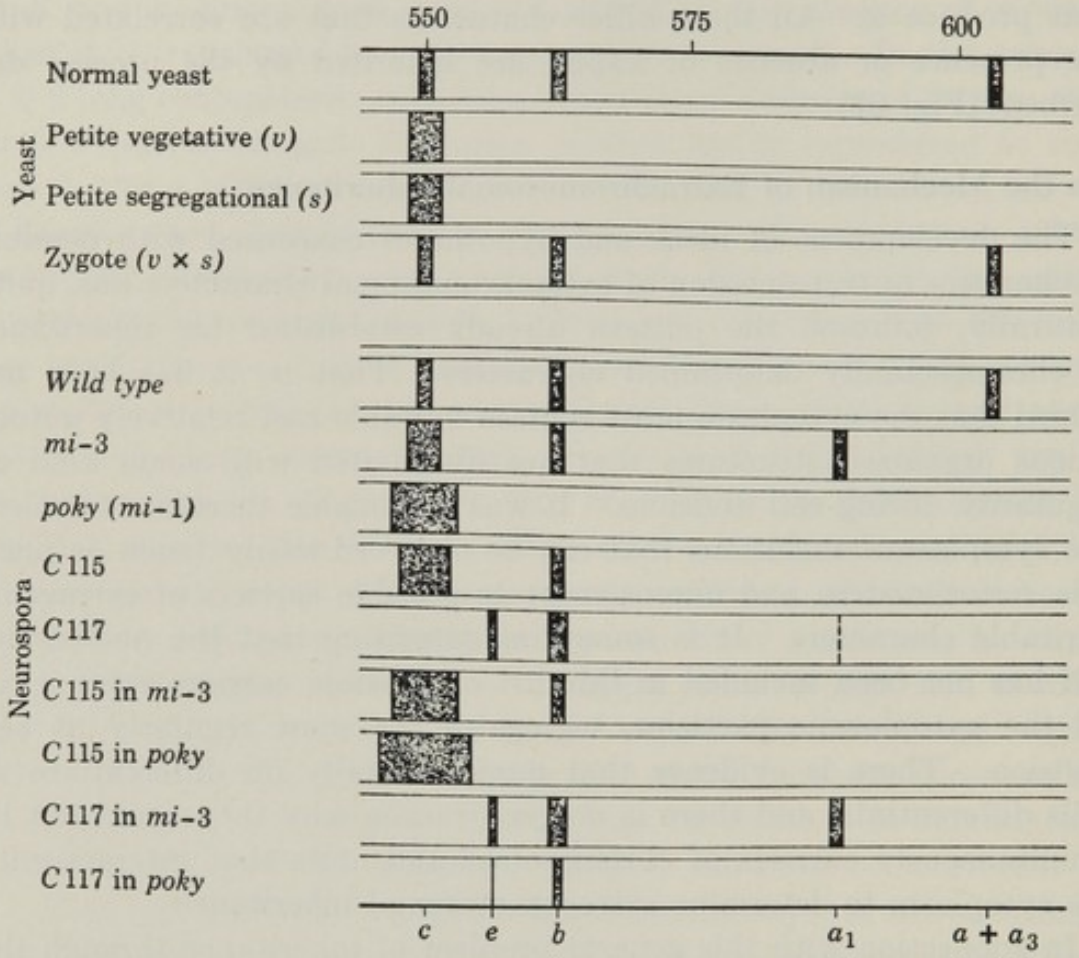


Fig. 98. The cytochrome components of a number of strains of yeast and *Neurospora*. The α absorption bands of the cytochromes are indicated with the wider bands representing higher concentrations. Actual differences are larger than indicated (see text). Positions of bands of some known cytochromes are indicated at the bottom of the figure as cytochromes *c*, *e*, *b*, *a*, and $a + a_3$.

mother cells, and in these cases cytoplasmic inheritance tends to be less clear-cut.

In corn or *Oenothera* the plastids have been involved since they change in color whereas in yeast and *Neurospora* it has been shown that the cytoplasmically inherited characters are accompanied by changes in the cytochrome systems that are carried by mitochondria (see Fig. 98). The killer character in *Paramecium* is also associated with the observable cytoplasmic kappa particles. In this case the change is so extreme that the particles are not observed at all in

sensitive strains, and kappa is not produced unless some is present in the cell. There is also a correlation between the expression of the killer phenotype and the quantity and quality of kappa as noted previously.

These facts and others suggest two principal alternatives with respect to the mechanism of cytoplasmic inheritance. (1) Any of the several recognizable types of cytoplasmic particles may have the continuity and stability to act as a determiner of heredity. These include plastids, mitochondria, microsomes, and perhaps other less well-known units. Virus-like infective agents might also be included. In this case transmission of hereditary characters can usually be assured by segregation of particles in perhaps a random fashion at cell division. Presumably a new cell would require only one particle of many contained in the mother cell in order to start replication and continuance of the expression. (2) Cytoplasmic inheritance should be considered in terms of the metabolic state existing in the cell as a whole. (See reviews by Sonneborn and Ephrussi (170, 172, 575).) Here it is necessary to assume that more than one stable state of metabolism can exist within the total metabolic potentialities of one genome. Transmission of a character in this case would then depend on the transfer at cell division of a pattern of function including various large cellular particles, enzymes, and metabolites. According to this scheme the specific properties of the cellular particles, the appearance and activities of which may be different in different cytoplasms, are determined by the interrelated chemical activities of the cytoplasm. This principle is in accord with the known facts concerning the dynamic state of metabolism. That is, the components of the cytoplasm exist in a state of constant flux characterized by a continuous degradation and resynthesis. This is known to be the case for many but not all components of the mitochondria and microsomes as well as for some components of the nuclei.

It is not possible to make a choice between these two alternatives or even to be certain that they are mutually exclusive. Still, there are a number of experimental facts that should be taken into account even though it is not feasible to choose between cause and effect at the present time. These facts deal with examinations of the effects of cytoplasmic inheritance on a variety of metabolic activities and general biochemical characteristics of cells, and by this approach it has been established that in several cases changes of many kinds accompany observed alterations in the large cellular particles. Little

work of this kind has been done with maize or *Oenothera,* but these should provide good material for investigations of this kind.

Work with *Epilobium* (410) has been less limited in outlook from the beginning, since morphological, physiological, and metabolic disturbances rather than particle appearances were often used as criteria for detection of cytoplasmic inheritance. The great variety of criteria used has already been mentioned (p. 324). Some representative and more specific data are given in Table 47. The reciprocal crosses

Table 47. A Comparison of Hybrids from Reciprocal Crosses among Several Races of *Epilobium* *

Figures are percentages and $= \frac{\text{hybrids of column 1}}{\text{reciprocal hybrids}} \times 100.$

Hybrids from	Plant Height	Water Content	Peroxidase Activity	Catalase Activity
Afghanistan ♀ × Jena ♂	97–110	—	122	91
Tubingen ♀ × Jena ♂	69–86	100	54	—
Stüttgart ♀ × Jena ♂	33–43	97	37	14
München ♀ × Jena ♂	31–40	97	37	36
Cambridge ♀ × Jena ♂	ca. 27	93	19	25
Lisbon ♀ × Jena ♂	ca. 10–15	—	12	10
Cäen ♀ × Jena ♂	ca. 5	—	8	—

* Data from Ross (521*a*).

given here were made with a number of geographical races of *Epilobium hirsutum,* using the Jena stock as a tester strain for comparative purposes. As shown in the table there is a rough parallel between plant height and the activities of peroxidase and catalase in the tissue. These values themselves vary enormously in the reciprocal crosses. That is, Afghanistan × Jena give quite similar values regardless of which is used as the maternal parent whereas Cäen × Jena give extremely different hybrids.

These data are in accord with the general theme propounded by Michaelis (410) that it is the entire cell that must be taken into account in cytoplasmic inheritance. If this is so, then the problem is one of understanding metabolic interrelations on a much more grand scale than is known at present. It is a simpler but not a more justifiable viewpoint to attribute cytoplasmic inheritance to the action of observably different cytoplasmic particles, and even though most investigators have recognized that such a change can be as much a result as a cause of the phenomenon, only a little effort has so far

been directed toward investigations of over-all metabolic processes or changes in systems other than the one that was discovered first. This is particularly true in the cases of plastid inheritance in the higher plants with the exception of *Epilobium*.

Attention has been given to biochemical changes that occur in *Paramecium* that can be correlated with the presence or absence of the killer character (564). As shown in Table 48 these differences deal

Table 48. Some Biochemical Properties of Killer and Sensitive Strains of *Paramecium* *

Measurement	*KK* (killer)	*KK* (sensitive)	*kk* (sensitive)
Oxygen uptake (mμl/animal/hr)	0.9	0.51	0.52
Increase in oxygen uptake with glucose (% of control)	92	130	154
Inhibition of respiration with streptomycin-5 mg/ml (% of control)	123	—	66
Inhibition of respiration with azide $2.5 \times 10^{-3}M$ (% of control)	89	29	60
Cytochrome oxidase (mμl/animal/hr)	0.026	0.069	0.075

* From Simonsen and Van Wagtendonk (564).

with respiratory systems, but only in a general way. The data demonstrate that appreciable differences between killers and sensitives do exist. It is particularly significant that respiration in the killer strain is relatively little affected by addition of glucose or azide. The azide would be expected to deactivate cytochrome oxidase, and indeed this strain has a relatively low cytochrome oxidase activity. It is thus clear that the respiratory patterns of killer and sensitive strains are significantly different, but the relation of this to the presence or absence of kappa is not known. The data indicate that the killer strain has either a lower concentration of the cytochrome terminal oxidase system or that this system is abnormal as is shown to be the case in cytoplasmic inheritance in yeast and *Neurospora*. In these latter two organisms, experiments have shown that cytoplasmic inheritance is accompanied by a variety of changes in chemical composition and thus in patterns of metabolism.

Particular attention has been given to properties of the mitochondria and especially to the cytochrome systems that are carried by these particulate bodies. One good reason for this is that the mitochondria are easy to isolate for the determination of enzyme activities, and the cytochromes can be observed directly on intact tissue by

means of a spectroscope. A summary of cytochrome compositions of a number of yeast and *Neurospora* strains is given in Fig. 98. Some of these characteristics are inherited cytoplasmically and some chromosomally as indicated (426, 429). These data demonstrate that somewhat similar and very striking changes in the composition of mitochondria can occur as the result of either extragenic or genic changes. That systems resulting from these two kinds of changes can interact to give a normal phenotype has been demonstrated in yeast by crossing petite-vegetative with petite-segregational. The diploid zygote thus formed contains a normal and a mutant allele of the gene in question and a mixture of cytoplasms. The resulting interaction produces normal mitochondria with respect to the cytochromes as shown in the figure. This indicates that different functions which contribute to, and control, the composition of the mitochondria are affected in the two strains of yeast, since each makes up for a deficiency in the other.

In *Neurospora,* mixed cultures which should give rise to heterocaryons containing mixed nuclei and cytoplasms have been observed to give either a *poky* or a wild phenotype. This is of particular interest in connection with interactions between the strains that show cytoplasmic inheritance (218). Proof of heterocaryosis was established by use of marker genes, but cytoplasm mixing can only be inferred from the fact that hyphal fusions and mixing of cytoplasms and nuclei are known to occur in the mold.

These results suggest that the cytoplasmic characteristics of the slower-growing strain are sometimes dominant over that of the more rapidly growing strain. A reasonable biochemical basis for this, for the *poky*-wild type combination, is provided by studies of the properties of isolated mitochondria from these strains. It was shown that the *poky* particles, in contrast to those from wild type, possess a potent enzyme system that destroys the cytochromes (cytochromase). This is due in part to a proteolytic action, and indeed these particles from *poky* digest virtually all their own proteins in a short time whereas wild-type particles remain relatively unchanged under the same conditions (37°C at *p*H 7.0) (418). In mixtures the *poky* particles digest those from wild type as well as themselves. If this proteolytic action occurs to any appreciable extent *in vivo* then it is not surprising that the *poky* character can be dominant over that of wild type. Actually the non-particulate fractions from both *poky* and wild type contain a low molecular weight organic compound that is inhibitory to the proteolytic action shown by the isolated *poky* part-

icles. Thus, the *in vivo* reaction is slow enough to permit an accumulation of cytochrome *c* in *poky*, but it may be fast enough to have a strong influence on the character of the cytoplasm in mixtures of wild type and *poky*.

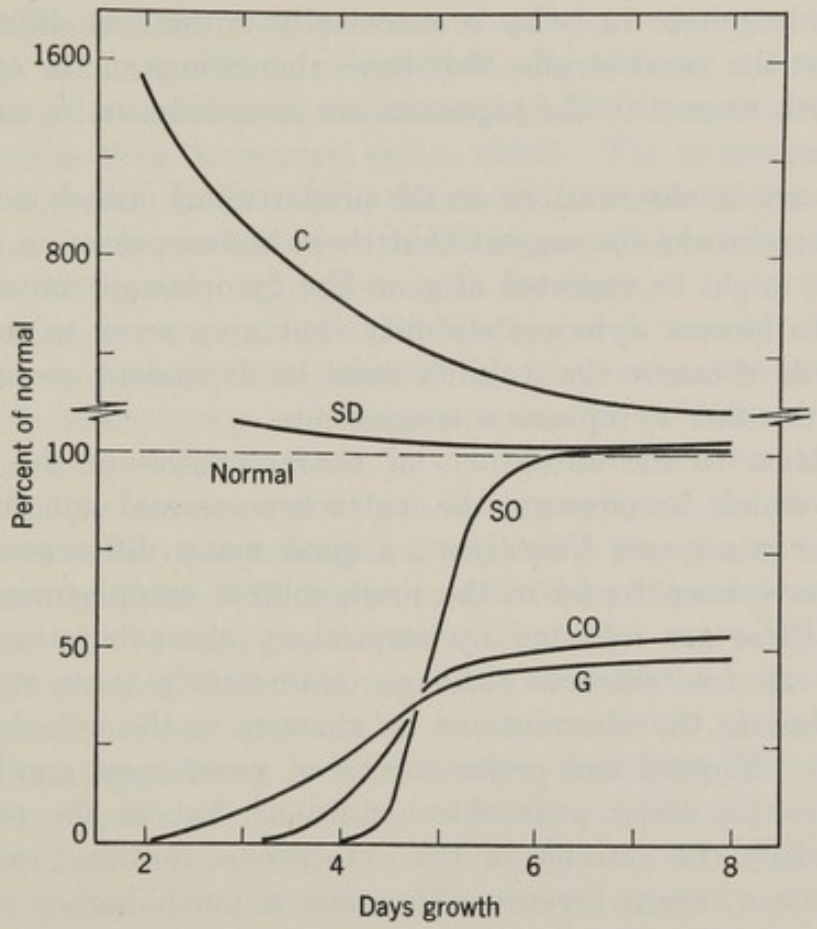


Fig. 99. Diagram showing relative growth (*G*); cytochrome *c* (*C*); succinic dehydrogenase (*SD*); succinoxidase (*SO*); and cytochrome oxidase (*CO*), levels of *poky* as compared to wild-type *Neurospora*. The wild-type characteristics are represented by the horizontal dotted line at 100%.

The large particles from *poky* differ from those of wild type in a number of ways in addition to the proteolytic capacity. As isolated from young cultures they contain an excess of flavin-adenine-dinucleotide, and they are deficient in RNA and polysaccharide (655). They are also abnormal with respect to the components of the succinoxidase system as expected from the observed cytochrome pattern of the whole mold. It is an interesting point, however, that during growth of *poky* the mitochondria change in composition and in the activity of the succinoxidase system (264). These changes are summarized in Fig. 99. As the mold culture ages the succinoxidase sys-

tem becomes more nearly normal, but this condition is never achieved. At the same time the cytochromase reaction of isolated particles drops below the detectable level. This whole process of change is repeated if a small inoculum is taken from an old culture, but there is much less lag with a large inoculum. As shown, the activity of succinic acid dehydrogenase in *poky* is essentially normal at different ages. In contrast the yeast strains that have aberrations in the cytochrome system with respect to the pigments are also deficient in succinic dehydrogenase.

These various observations on the properties of mitochondria in the different strains do not suggest that these bodies possess a static stability that might be expected of gene-like cytoplasmic units, but they do seem to have a dynamic stability that may serve in inheritance. In this case of course the stability must be dependent on rapid interaction with other cytoplasmic components.

In addition to the alterations of characteristics of the large cell particles which accompany the extrachromosomal inheritance described for yeast and *Neurospora* a good many differences in composition have been found in the more soluble cytoplasmic fractions. Some of these are reflected by respiratory characteristics of whole cells and cell free extracts although qualitatively these studies simply corroborate the observations on changes in the cytochrome system (654). Normal and petite strains of yeast show similar respiratory activities under anaerobic conditions, but in the presence of oxygen, where the activity of the cytochrome terminal oxidase system is needed, normal yeast cells show a much higher respiratory activity, and this is inhibited by cyanide or azide.

The situation is similar with regard to the *poky* and wild-type strains of *Neurospora* except that in this organism aerobiosis is required. The respiration of young but not old *poky* is not inhibited by cyanide or azide, and the rate of oxygen uptake is about one-third that of wild type. The normal strain is inhibited by cyanide or azide to the extent of about 80%. The *mi*-3 strain of *Neurospora* is like wild type in that respiration is inhibited by cyanide, azide, or carbon monoxide, but it is more sensitive to the inhibitors than the wild strain. In addition, the properties of the terminal oxidase system, which evidently depends on cytochrome a_1 for coupling to oxygen, do not change significantly during the growth of a culture as is found with wild type but not *poky*.

In addition to these respiratory differences that accompany the cytoplasmically inherited changes, observations have been made on

concentration or enzyme activity changes with respect to a variety of cellular components. In yeast, besides the enzyme differences between normal and petite that have already been noted for aerobic cultures, petite is extremely deficient in α-glycerophosphate dehydrogenase and DPN-cytochrome *c* reductase activities, it contains a new DPN-independent malic-cytochrome *c* reductase activity and a twofold excess of lactic acid dehydrogenase. In addition it contains from 2 to 5 times less aconitase, fumarase, and DPN-isocitric dehydrogenase activities than the normal strain (280). The larger part of these enzymes is found in the soluble fractions from yeast, but an appreciable quantity is associated with the large cell granules, and in petite the activities are reduced in both fractions.

Information on the relative distribution of a variety of enzymes, not including most of those studied in yeast, indicates differences in activities between *poky* and wild-type strains of *Neurospora* of up to twofold for several of them, but it has not been established that these variations are not due to non-homogeneity of the strains tested with respect to chromosomal genes. Alkaline phosphatase appears to be consistently about 50% higher in different *poky* reisolates. It has been found that young *poky* contains about 30% more DNA per unit dry weight of mold than wild type, but none of this was detected in non-nuclear fractions so there is no indication of the existence of DNA-containing particles in *Neurospora* cytoplasm. Ribonucleic acid and polysaccharide are found in about the same total concentration in *poky* and wild type, but the distribution in the cells is different. The large granules or mitochondrial fraction of *poky* are relatively deficient (twofold or more) in these components. On the other hand *poky* contains approximately twice as much riboflavin and niacin as wild type, the excess of niacin being found in the soluble fraction whereas the riboflavin (as FAD) is relatively concentrated in the mitochondria.

It is clear from these various data on the relative concentrations and distributions of cellular components of yeast, *Neurospora, Paramecium,* and *Epilobium,* that a characteristic of cytoplasmic inheritance is a redistribution of concentrations of a variety of metabolites and catalytic activities. This is evident even though really extensive and systematic investigations remain to be done in these organisms, and very little has been done along these lines in many other species in which extrachromosomal inheritance has been demonstrated. Such investigations are highly desirable if only from the standpoint of providing a more complete description of the phenomenon. They can-

not in themselves yield an explanation of the mechanism of cytoplasmic inheritance since such differences in concentrations of cellular components can be either the cause or the result. It is much more likely that an understanding of the mechanism of this type of inheritance must come from studies of the process itself but by means that take into account the over-all metabolic differences that exist between strains that are characterized by showing extrachromosomal inheritance.

The Induction of Cytoplasmically Inherited Characters

A good many of the known examples of extrachromosomal inheritance are of spontaneous origin, and little can be said as to the cause. It is presumed that those cases demonstrable through interspecific crosses as in *Epilobium* and *Oenothera* arose through the effects of slowly diverging genomes in different isolated populations. Thus, the character of the cytoplasms could have changed in such small increments, as the result of successive spontaneous mutations of chromosomal genes, as to seem almost continuous. The observations on the many degrees of manifestation of cytoplasmically inherited phenotypes in reciprocal crosses between different geographical races of *Epilobium hirsutum* are highly suggestive of the occurrence of a process of this kind.

The strains *poky* and *mi*-3 derived in *Neurospora* were obtained from crosses involving aged cultures of wild-type strains. There is no evidence for any significant difference in the genomes of the slow-growing strains and the genomes of the wild strains from which they were obtained, so it would appear that these changes in cytoplasmic characteristics occurred fairly rapidly without any great influence by the chromosomes. However, as shown in yeast, a 15-fold difference in the rate of spontaneous change from normal to the petite-vegetative character has been shown to segregate as though this tendency for change is influenced by a particular chromosomal gene. It is therefore indicated that certain genomes are more favorable for the occurrence of cytoplasmically inherited changes, as shown in yeast and in corn.

Informative data on induction of cytoplasmically inherited characters were obtained by studies with yeast (172) and with *Paramecium* (37). It has been demonstrated that the change of normal baker's yeast to the petite-vegetative phenotype can be induced in all the buds derived from a single cell by isolations in the presence of appropriate concentrations of euflavin (172). Brief treatments with this

substance can give rise to either normal or petite cells each of which in turn can produce both normals and petites. This suggests that the acridine derivative induces an unstable state in the cytoplasm—a state which can then change further in the direction of the stable normal phenotype or the stable petite phenotype. These experiments, which demonstrate the influence of a foreign chemical compound on yeast cytoplasm, suggest that environment is an especially important factor in the induction of cytoplasmically inherited characters. This conclusion is in accord with the results derived from investigations of the effects of environmental changes on the antigenic specificity of cilia in *Paramecium*. Some details of these experiments are presented in the chapter on environment (p. 295). In this case it is clearly demonstrated that antigenic specificity is inherited cytoplasmically (and conditioned by chromosomal genes) and that these characteristics can be altered by a variety of environmental changes including those of temperature, nutritive requirements, radiation, and specific antibodies.

Considering all these results it appears that cytoplasmically inherited characters can be induced by instituting conditions that change reaction rates of systems relative to the rates of other systems. If the conditions imposed permit readjustment of the whole cellular metabolic pattern to a new stable dynamic state that is favorable to survival and reproduction of the cell then the new system can persist. This interpretation of the data which indicate the nature of cytoplasmically inherited characteristics still does not include a description of a generalized mechanism of the process of the inheritance. We consider that this process involves the segregation, at cell division, of metabolic units having a sufficient influence within the inherent capacities of a given genome to cause the stabilization of a particular pattern of metabolism. These units, so far as we know, have the size limits extending from those of the smallest molecules to the size of the whole cell.

CHAPTER 13

The Problem of the Mechanism of Development

Whether they are unicellular or multicellular, and whether the environment is constant or in continuous flux, all organisms undergo a process of orderly change or development during the course of their lives. The developmental state achieved at any particular time during the process is generally described as the phenotype. The usage of the term phenotype in the present context is the usual one, but it is dictated by expediency and describes only the condition at any one time of a system in continuous change. An analogous situation might be the description of a mathematical equation by the slope of its curve at any one point rather than giving the complete curve. No mathematician would attempt to do this, any more than a biologist should try to understand a phenotype resulting from a particular set of genes without making a careful study of the development of that phenotype. The study of development must not only include a descriptive analysis, but, more important, an analysis of the mechanism whereby the organism develops a particular phenotype. Both types of analysis require an invasion into the field of embryology on the part of the geneticist.

In their purely descriptive phases embryology and genetics are two distinct disciplines: embryology describes the patterns of development which occur, and genetics describes the mechanism of inheritance of these patterns. The two sciences meet in the search for the causality of development and the mechanism, and it is at this point that both seemingly meet an impasse. The nature of the impasse has been clarified somewhat by the gene theory with its preformationistic basis, and redefinition of epigenesis as the expression or result of gene action. But the impasse still exists in that the primary nature of gene

action is an unsolved problem, and the nature of the interaction of the presumed gene products in the nucleus and cytoplasm is as yet in the domain of speculation. These must be the basic factors underlying development, but there is considerable ignorance as to how they work.

On the assumption that it will be profitable to consider development in order to understand its underlying causes better, an attempt will be made here to consider those aspects of the mechanism of development which seem germane to the problem of gene action as that problem is presently understood.

1. Aspects of Development

The developmental process may be divided into three components: differentiation, organization, and growth. These, it must be emphasized, are not independent processes, but aspects of a total process dedicated to the elaboration of a complete, functional organism.

The first of these component processes, differentiation, is perhaps the most often emphasized in embryological research. It involves progressive changes in cell structure and chemistry which lead to the formation of different tissues. Although the term is most often used in connection with multicellular organisms, in the broad sense, even unicellular organisms show differentiation in function and structure in the cell during the life of the individual.

In the developing organism differentiation is accompanied by organization or localization of differentiated cells leading to morphogenesis, or the establishment of a definite pattern of structure. It is usually, but not invariably, accompanied by growth, a process which will be defined here as increase in mass of protoplasm with or without increase in number of cells.

If the action of genes is considered in the light of the three principal aspects of development that have been adumbrated, it becomes evident that genes have a number of functions. They must control differentiation, for the types of cells and tissues produced are unique for each type of organism; they must control the organization of parts, for structure and form are inherited; they must control growth, for it is obviously not an uncontrolled process, but an ordered one resulting in various sizes and shapes of organisms, and in organs and parts of regulated size.

The Effects of Differentiation

The process of differentiation in the multicellular animals and plants is probably one of the most spectacular aspects of development. Superficially it involves the formation of distinctly different types of cells by mitosis from a zygote mother cell. Thus the nerve, muscle, epithelial, etc., cells of an animal, or the cortical, phloem, and mesophyll cells of a plant are all derived from a zygote. Differentiated cells are not only morphologically different, but, as befits their different functions, chemically different as well. These chemical differences are manifested at all conceivable levels, physiological, constitutional, nutritional, and enzymatic, as well as response to environmental factors such as oxygen concentration and *p*H. An appreciation of the magnitude of some of these differences is necessary to the understanding of differentiation. Some of them are therefore discussed below in detail.

It has long been known that the culture of tissues *in vitro* requires careful attention to the nutrient medium, since tissues differ in their nutritional requirements both qualitatively and quantitatively. This is as true for adult tissues as for embryonic tissues which are in the process of differentiation. Spratt (586) has shown that chick embryo heart tissue will develop at very low concentrations of glucose whereas nervous tissue will not. Almost twofold more glucose must be supplied to nerve tissue before development will proceed. These differential nutritional requirements are, of course, indications of basic metabolic differences among tissues—a fact more directly demonstrated by results from inhibition studies. Iodoacetate, malonate, cyanide, and azide inhibit the growth of chick brain but have little effect on the heart. Fluoride, on the other hand, inhibits the heart but not the brain (587). One interesting result of these investigations on embryonic chick tissue was the demonstration that the embryonic tissues respond in the same way as the adult tissues to the nutritive sources and inhibitors. From this the tentative conclusion may be drawn that the metabolic pattern of the early differentiating tissue is similar to the metabolic pattern necessary for its maintenance in the adult (582, 586, 587).

It can be assumed that metabolic differences reflect quantitative and perhaps qualitative enzymatic differences. Table 49 presents data which show the correctness of this assumption. The enzymatic activity in various organs of the same animal is quite different for most

Table 49. Relative Enzyme Activity Values for Various Tissues of Rat, Mouse, and Carp

	Liver	Kidney	Skeletal Muscle	Spleen	Brain
Aldolase, rat	12,100	7,800	74,000	4,800	15,800
D-Amino acid oxidase, rat	15–13	108–132	—	—	—
Glutamic-oxaloacetic transaminase, rat	245	245	316	16	260
Ribonuclease, rat	0.37	1.63	—	2.06	—
Aconitase, rat	62	80	—	—	10
Thiaminase, carp	2.5	1.34	0	25	0.21
Peptidase, rat (glycylde-hydroalanine substrate)	60	1,620	—	—	—
Catalase, mouse	8.00	3.20	0.01	0.12	0.00
Lactic dehydrogenase, mouse	428	369	972	144	228
Cytochrome *c*, rat (μg/mg dry weight)	0.607	1.433	0.381	0.05 (skin)	—

enzyme types. In certain cases more precise determinations of enzyme presence than activity are possible when a known prosthetic group can be determined quantitatively by direct methods. Some examples of the variations in concentrations of cofactors are given in Table 50.

Table 50. Concentrations of Some Important Cofactors in Rat Tissues

	Liver	Kidney	Skeletal Muscle	Brain
Flavin-adenine dinucleotide, rat (mg/kg wet weight)	45	20	—	—
Adenosine triphosphate rat (mg P/100 grams of tissue)	0.5–1.5	2.7	40–50	9–12
Phosphocreatine, rat (mg P/100 grams of tissue)	6–10	2.5–4	40–50	1.5–7.5

The differences in synthetic capacity expected as a result of changed metabolism of differentiated cells is manifested in form, chemical constitution, physiological response, secretory ability, etc. Although certain differences in content among cells may be ascribed to some absorbing and storing compounds synthesized by all, it is obvious that many are unique sources of certain compounds. For example, the production of hormones in an animal is the function of the endocrine

glands which produce specific hormones with specific physiological properties. The capacity to respond physiologically to these hormones, at least to a recognizable degree, is again specific for certain types of tissues. Thus secretin produced by the small intestine mucosa in the vertebrates apparently affects only the pancreas, inducing it to discharge digestive enzymes despite the fact that secretin is widely distributed throughout the body soon after food enters the small intestine from the stomach.

Antigenic differences existing between different individuals have been discussed in Chapter 6, but no reference made to the antigenic heterogeneity which may exist within the individual. Actually it has long been known that the antibodies induced in a host animal by antigenic components from an unrelated donor show considerable differences, depending on the type of tissue from which the antigens are extracted. Although each individual seems to have some antigens common to all tissues, organ or tissue specific antigens are also produced. These are specific not only in the sense that they are found only in extracts from a particular organ, but their antibodies show a definite, specific effect on the same organ. Ebert (157) and Weiss (682) have demonstrated such effects in developing chicks. Ebert has shown that antibodies produced in rabbits by extracts from spleen, heart, and brain tissue from chicks have differential effects on the development of the chick blastoderm. Anti-spleen and heart sera affect primarily the mesodermal elements whereas the brain antisera prevent nerve tissue development. Anti-spleen and heart sera have different effects on the mesodermal derivatives, for a heart may develop normally in the blastoderm in presence of anti-spleen serum but not when anti-heart serum is present.

These observations demonstrate the considerable chemical and physiological divergence among tissues that results from differentiation. The differences, so striking among adult tissues, are evident even in the very early embryo, where, although the morphological differences may not be great, chemical differences first appear. Some of the antigenic differences appear very early in the chick embryo, and certain organ specific antigens are detectable even before the primordia of the organs with which they are associated make their appearance (157).

Quantitative enzymatic differences also become evident early in development when enzymes begin to assume the levels of activity found in the adult tissues. Moog (437) in her investigations on the distribution of alkaline phosphatase in embryonic chick tissues in dif-

ferent stages of development has given clear evidence of this point. As shown in Fig. 100, the level of activity of this enzyme is relatively uniform in "undifferentiated tissue." As tissues begin to differentiate, however, there is a rise or fall in the base undifferentiated level in each tissue as it begins to become separate and distinct from the undifferentiated mass. The change in the level of activity in each tissue is in the direction (high, low, or intermediate) of the

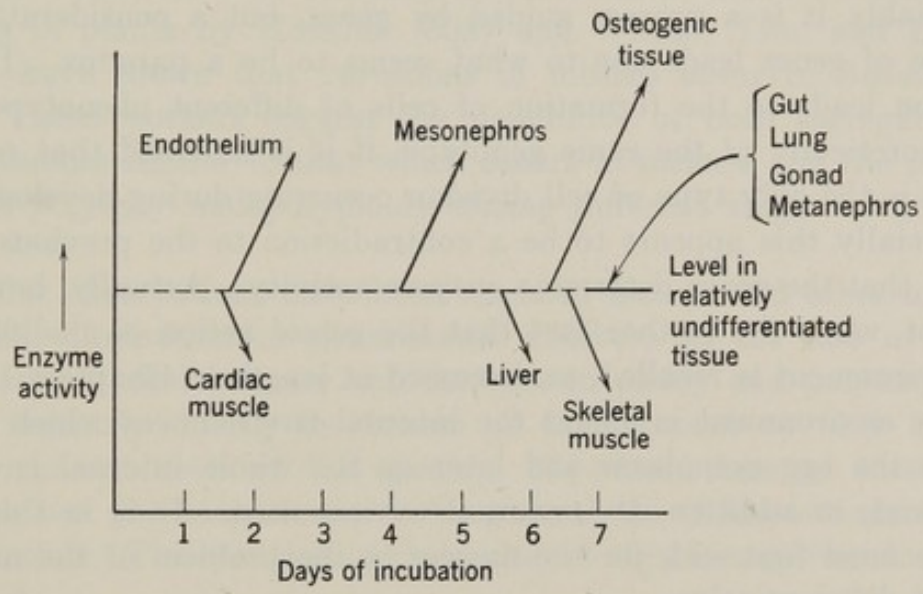


Fig. 100. Changes in alkaline phosphatase activity during the first week of incubation of the chick embryo. The arrows indicate the direction of change of enzyme activity from the level found in the very early embryo and in relatively undifferentiated tissue. After Moog (437).

level found in the mature tissue. Enzymes known to be important in the functioning of a tissue in the adult may appear at the proper levels in that tissue long before the tissue is called upon to perform its role in the adult. Alkaline phosphatase is always present in high concentration in intestinal mucosa where it is concerned with the digestive and absorptive functions. In the embryonic chick it begins to increase in concentration in the intestinal mucosa during the third week of incubation and surpasses the adult concentration by the time of hatching so that the emerged chick is well prepared to cope with food entering through the digestive system.

Hence it is seen that as part of the process of differentiation an enzyme necessary in adult function is supplied at the proper level before the tissue or organ is called upon to perform that function. Although all these results are to be expected, since enzyme systems are basic to tissue function and must develop as the tissue develops

as part of the tissue, they are nonetheless important as partial confirmation of what otherwise would be an hypothesis.

Regional differences in morphological and physiological properties probably only arise as a consequence of prior chemical differentiation (668). Thus differentiation must be looked upon as a process of producing regional differences in metabolism which lead to secondary morphological and physiological differences. The problem then immediately arises, how does this regional organization come about? Presumably it is a process guided by genes, but a consideration of the role of genes leads one to what seems to be a paradox. Differentiation leads to the formation of cells of different phenotype, but yet theoretically of the same genotype, if it is assumed that regular mitosis is the only type of cell division occurring during development. Superficially this appears to be a contradiction to the previous affirmation that the genes determine enzyme activity. Actually, however, it is not, when the further fact that the genes' action is modified by the environment is recalled, as discussed at length in Chapters 10 and 11. By environment is meant the internal environment which exists first as the egg cytoplasm and later as the whole internal environment, and, in addition, the external environment. It is in this fact that we must first seek for the answer to the problem of the mechanism of differentiation.

Capacities of the Nuclei during Development

The assumption is generally made that the cell divisions occurring during development are true mitoses giving rise to daughter cells with identical chromosome constitutions. It would follow, then, that the nucleus of the most highly differentiated cell should be no different from the zygote from which it is derived. There is indeed enough observational and experimental evidence to indicate that this generalization is a fairly close approximation to the truth provided that the definition of degree of difference existing among nuclei in the same individual is qualified to take into account certain of the factors discussed below.

The fact that the somatic chromosome number is almost always the same as that found in the zygote and double the number found in the gametes is in itself significant evidence for the regularity of true mitosis during development. Some variations in this general rule exist, however, and it is difficult to determine always which are significant and which insignificant deviations.

Hsu and Pomerat (306) have demonstrated that somatic cells of mammalian tissues may have more or less chromosomes than the diploid number usually assumed for the species. But since the variations are not regular, as far as can be determined by the preliminary observations, and appear to be at random with the average number for all tissue types being the assumed diploid number, Hsu concludes that the variations are not significant. Mammalian tissues are not, in other words, different basically in chromosome number. Studies of mitosis in plants by Huskins (308) and Wilson, Tsou and Hyypio (696), have shown that variations in mitosis occur in *Allium* root tips. These workers suggest the possibility of both segregation of chromosomes similar to that which occurs in meiosis and the production of polyteny and polyploidy during different stages of development.

Similar observations have been made in insects and other animals in which diminution of chromosome number, by the loss of whole chromosomes, and increase and decrease in ploidy and polyteny may occur regularly during the course of development at very definite stages characteristic of the species. However, none of these phenomena has yet been shown to be general, or of importance in differentiation. The question of their general significance remains to be clarified.

Many experiments have been performed to determine by mechanical manipulation whether nuclei present in segmenting eggs are totipotent, or capable of continuing to control the process of normal development when moved from one part of the early embryo or cleavage stage to another. These experiments date back to the classical work of O. Hertwig (276) and Spemann (578) with the early stages of the amphibian embryo. By means of pressure applied unequally to the egg Hertwig altered the normal course of cleavage in the frog's egg so that the nuclei resulting in cleavage occupied different parts of the segmented egg than they would have if segmentation had been allowed to proceed undisturbed. No effect was noted on the embryos from eggs treated in this fashion. All developed normally to completion. Spemann applied a ligature to a newt egg before cleavage in such a way that one half contained a nucleus and the other half did not. The two halves were connected by a bridge of cytoplasm. He then demonstrated (1) that cleavage did not occur in the enucleated half, but did so in the nucleated half, and (2) that nuclei from the half undergoing cleavage occasionally escaped through the cytoplasmic bridge to the undeveloping, enucleated half. Even if these

nuclei were many generations removed from the original zygote nucleus they started cleavage in the enucleated half which culminated in the production of a complete, normal embryo.

Experiments similar in intent, and applying the technique of destroying nuclei in insect eggs undergoing cleavage by ultraviolet light, have shown that up to the 128-cell stage in the damsel fly, *Platycnemis,* the intact nuclei are capable of replacing by subsequent mitosis those destroyed, so that development proceeds undisturbed (556). More recently Briggs and King (75) have performed the critical experiment of removing nuclei from frog's eggs and replacing them with nuclei from cells of the blastula and gastrula stages. Such artificially nucleated eggs are capable of developing to completion despite the fact that they contain nuclei many generations removed from the zygote nucleus.

These experiments demonstrate conclusively that at least during the early stages of development the nuclei retain the capacity to direct the whole of development from the zygote stage. Successive generations of nuclei produced by regular mitosis are then interchangeable and hence totipotent. Two reservations must be borne in mind with respect to this broad generalization, however. First, there has been no demonstration as yet that nuclei from highly differentiated tissue such as adult epidermis, or muscle, are capable of acting as zygote nuclei. Hence there still remains the possibility of nuclei not continuing to be totipotent indefinitely. Second, the establishment of totipotency of a nucleus taken from a differentiated tissue does not prove that the nuclear contents of all tissues are identical with respect to the functions carried out in those tissues. Nuclei may be totipotent and still carry on different functions in the tissues of their origin. The issue is not whether the chromatin has different functions or appearance in different tissues but whether it loses in the course of development totipotent capacity. The answer at present seems to be in the negative. The Weismannian theory that development is the result of segregation of nuclear contents during cell division is therefore incorrect, and attention must be focused on the cytoplasm rather than the nucleus as the most immediate source of variability among somatic cells of the same individual.

Establishment of Differences Early in Development

Any regional chemical differences in the cytoplasm of the fertilized egg would not necessarily be obvious ones. Yet a chemical differen-

tiation within the cytoplasm could easily lead to immediate changes in gene action by the different regions of the cytoplasm becoming separated during cleavage. In this way the same genotypes derived by mitosis from a single zygote nucleus could conceivably be injected into different environments.

Animal eggs generally show a high degree of localization of cytoplasmic material which becomes especially marked after centrifuga-

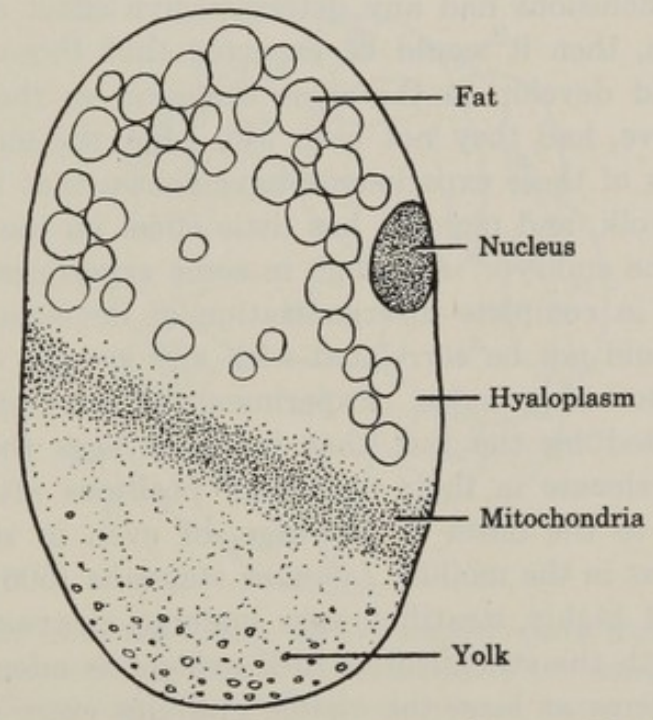


Fig. 101. Centrifuged egg of *Limnaea stagnalis,* a snail. After Raven and Bretschneider (506).

tion (Fig. 101). In most of them mitochondria are demonstrable, as in mature cells, and lipoid material may occur as droplets. In addition, eggs of many species contain pigment and yolk granules which may be distributed evenly throughout the cytoplasm, or localized in specific areas or bands. The peripheral portions of all eggs form a gel-like cortical layer which can be distinguished from the more liquid interior by a variety of techniques. In some animals the stiff cortex may be differentiated into morphologically or physiologically distinct areas which can usually be shown to have considerable importance in the future development of the egg. Examples of these cortical modifications are the gray crescent (see p. 368) of the amphibia, the somewhat similar yellow crescent of the ascidians, and the polar plasm of the molluscs.

Much attention has been focused upon the distribution of the visible cytoplasmic inclusions in the egg interior. Attempts have been made to correlate their frequently unequal distribution among the cleavage cells, or blastomeres with the course of development of the different blastomeres. Eggs have been centrifuged to force the visible inclusions into positions which they would not have occupied if the eggs had been left intact. This results in their being distributed, during cleavage, into blastomeres which they ordinarily would not occupy. If the inclusions had any determinative effect on the fate of the blastomeres, then it would be expected that those receiving the inclusions would develop in the same direction as those which lost them would have, had they not been lost. For the most part, however, the results of these experiments have shown that the redistribution of lipids, yolk, and pigment has little effect on the course of development of the embryo. Although in some experiments centrifugation did result in complete disorganization of development, the disorganization could not be correlated with any specific visible change in the distribution of granules. Experiments of this nature are sometimes complicated by the fact that in many eggs the cytoplasmic granules may relocate in their customary positions after centrifugation and prior to the onset of cleavage, or even in some instances after cleavage, as in the mollusc *Limnaea stagnalis* (506).

The fact that highly stratified eggs undergo cleavage and normal development with the stratification still evident is adequate evidence that in some forms at least the visible granules exert no determinative influence on the course of development by their localization. This is not meant to imply that they play no role in development, but only that their distribution is unimportant in any role that they may play. In this connection it should be noted that the cortex may play an important part in differentiation, which might not be influenced by centrifugation because of its stiff nature maintaining the organization necessary for development despite centrifugal forces.

Although the experiments described above give no direct evidence of cytoplasmic constituents being of any consequence in early differentiation, other observations and experiments of a somewhat different nature, although performed with similar intentions, have given conclusive evidence and proof for the significance of the cytoplasm in development. In the annelid worms and the gastropod molluscs (snails, etc.) the blastomeres formed during the early stages of development can be shown to have determined fates. The blastomeres are predestined in subsequent divisions to produce cells going into the for-

mation of specific organs or parts of the fully formed larvae. Cleavage takes place not only with a great deal of coordination among the dividing cells but with almost perfect spatial organization of the blastomeres relative to one another. The earliest blastomeres can be numbered and their descendants followed through the subsequent divisions up until tissues and organs are formed. It can then be established that specific blastomeres recognized by their position or even phys-

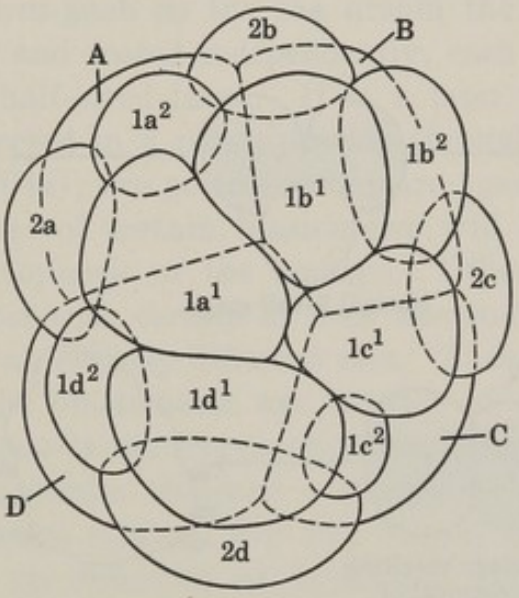


Fig. 102. The sixteen-cell stage of the annelid worm *Nereis*. After Wilson (694). The blastomeres are numbered according to the accepted scheme which indicates their derivation. The determined fates of some of these blastomeres are as follows: *D* will give rise to the mesodermal elements such as the coelomic lining, gonads, and longitudinal muscles as well as part of the alimentary canal. 2*d* forms the ventral nerve cord and the circular muscles. The cells marked $1a^2$, $1b^2$, $1c^2$, and $1d^2$ form the larval ciliated belt.

ical peculiarities go into formation of specific areas in the larvae, and thus the prospective fate of each cell can be mapped with a considerable degree of accuracy from early in development, as shown in Fig. 102.

Although these observations are of interest in showing the great degree of organization achieved during the early stages of development, they do not in themselves prove that the blastomeres are irrevocably destined to fulfill certain functions in development. Their fate may be determined only by their position relative to one another and not by any intrinsic difference had by each initially. Experiments performed to test this important point have shown conclusively that in the Annelida and Gastropoda the removal or de-

struction of blastomeres causes drastic alterations in the course of development. The part that ordinarily develops from the absent blastomere or blastomeres does not develop and a defective larva or adult results. Figure 103 illustrates the result of removing the polar lobe of the egg of *Dentalium* during its first division. A larva completely devoid of mesoderm is produced. Less drastic effects can be obtained by removing blastomeres in later cleavage stages. Similar results

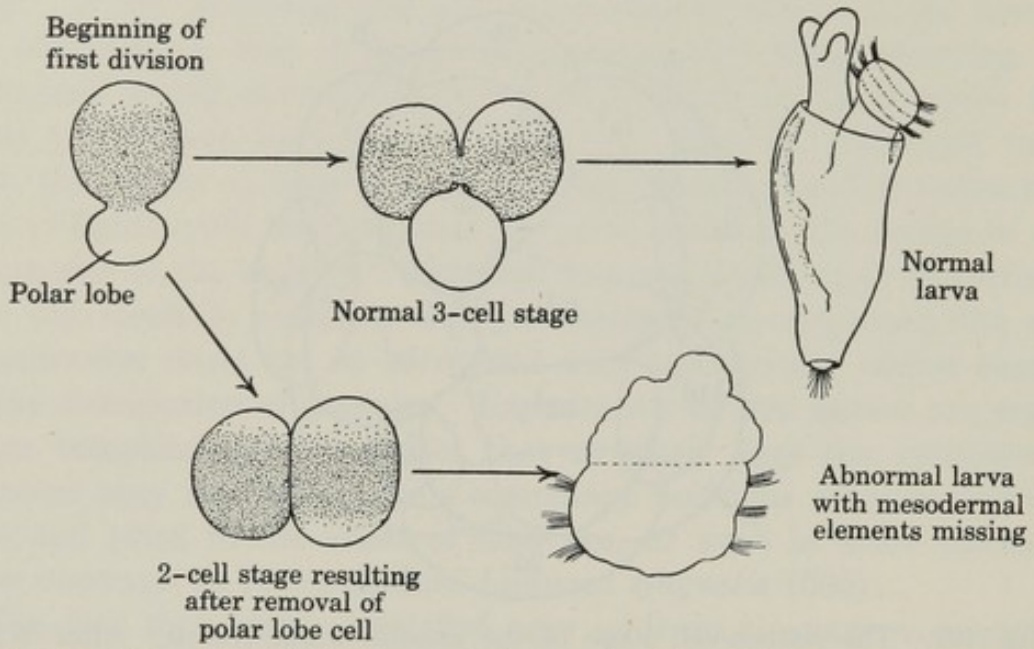


Fig. 103. The effect of the removal of the polar lobe of the egg on the development of the larvae of the mollusc *Dentalium*. After Wilson (694).

have been observed with a large number of other gastropods, Annelida, certain ascidians (sea squirts), and ctenophores (comb jellies). Isolated single blastomeres have also been observed to continue development, to divide and differentiate, but to produce only that portion of the embryo which would have been their fate had they remained part of the whole.

It is difficult to avoid the conclusion that in these invertebrates the blastomeres possess an inherent capacity to self-differentiate in a determined direction, and that the role of one of them cannot be assumed by another. At the time of commencement of cleavage the eggs of these forms must achieve a degree of organization which entails localization of materials and capacities. Cleavage presumably separates these heterogenous elements and thus the course of development of each part is determined. This may be taken to be proof

that differentiation is at least in part the result of unequal distribution of cytoplasmic metabolic capacities.

Investigations on the eggs and early developmental stages of other animals such as the Echinodermata and Amphibia give results which are subject to essentially the same interpretation provided that it is recognized that heterogeneity among blastomeres may be achieved in somewhat different ways and at different stages in some organisms than in those described in the preceding paragraphs.

If in an echinoderm such as the sea urchin the first two blastomeres are separated and reared independently, each will develop into a perfectly formed half-sized larva. If in a later stage of cleavage the cell mass is bisected in a plane passing through the animal and vegetal poles (Fig. 104*a*), two perfect larvae are again obtained. Furthermore the removal of certain blastomeres will in no way affect drastically the development of the embryo. Other blastomeres are able to repair the loss with certain of their descendants which would otherwise carry out an entirely different role. Thus it is evident that in the sea urchin the blastomeres are capable of different types of development; their fate is not irrevocably determined. This is in direct contrast to the results obtained with the molluscan and annelidan eggs and cleavage stages. For this reason the sea urchin egg and its blastomeres are described as *regulative;* i.e., they possess the capacity to make up for the defects incurred by destroying or removing parts during development. They possess a degree of versatility of differentiation into various channels not demonstrated by gastropods and other invertebrates like them which start development from eggs organized into different areas of development patterns and hence have a *mosaic* organization. Actually the distinction between the regulative and mosaic types breaks down upon further analysis of those forms like the sea urchin and frog which exhibit the regulative type of organization.

If the sea urchin egg is divided in two by ligature in a plane perpendicular to the animal vegetal axis, and the half without the zygote nucleus fertilized, two perfect larvae are not formed. On the contrary, a larva with cilia covering the external surface and no endodermal derivatives results from the animal half—the so-called animalized larva (Fig. 104*b*). From the vegetal half there is produced generally an exogastrula (vegetalized larva) with an evaginated oversized gut, or a more nearly normal larva with a larger gut than usual (Fig. 104*b*). Thus it would appear that the animal half produces primarily

ectodermal derivatives whereas the vegetal half produces primarily endodermal material. Horstadius (299) has shown by a large number of experiments in which he recombined various animal and veg-

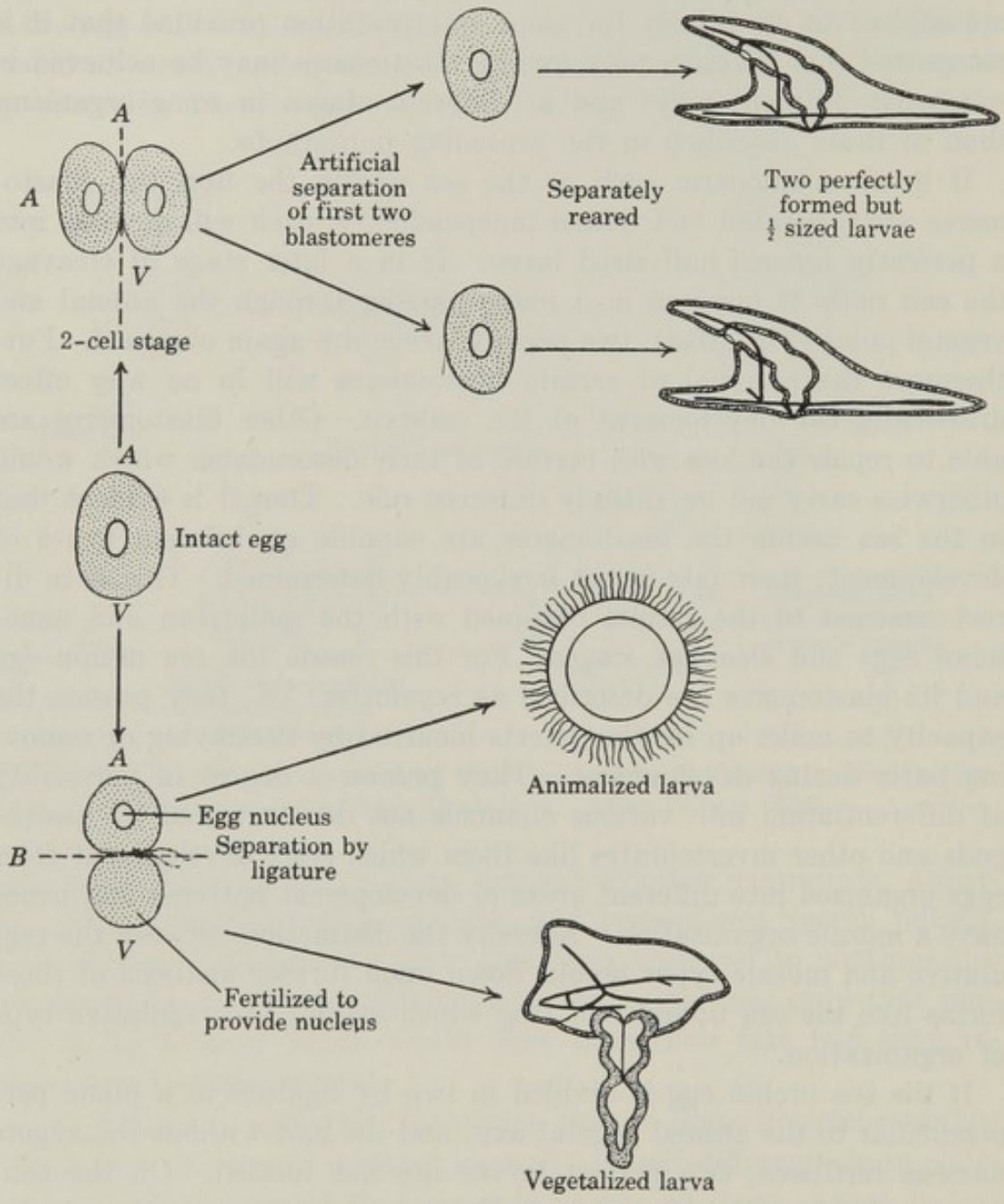


Fig. 104. The effects of the artificial division, in two different planes, of the sea urchin egg.

etal portions of the sea urchin egg that there are two forces operating in its development—the animal and the vegetal. He maintains with Runnström (523*a*) that in the intact egg the two are in balance, and a larva with the normal complement of tissues and organs re-

sults within the normal range of size so long as this balance is maintained intact. However, if the balance is upset by the removal of animal or vegetal material of the egg, the early cleavage cell mass, or the blastula, the course of development is swung in favor of the material present in most abundance. Presumably any part of the egg, even a small fragment, containing a nucleus, will develop into a near normal larva provided that both animal and vegetal material is represented (261).

Although the sea urchin egg is regulative to a large extent, it is quite clear that it also displays mosaic properties. Like the Annelida and the Gastropoda it does show a differentiation of cytoplasmic capacity from a very early stage of development in the obvious differences between the animal and vegetal areas of the egg.

Changes in the development of the sea urchin embryo can be brought about by treatment with chemicals. Lithium salts such as LiCl cause a normal egg to develop in the vegetal direction, producing an exogastrula similar to that developing from the isolated vegetal half of an egg (271). Certain amino acids such as proline and arginine also cause vegetalization (300). On the other hand, NaSCN and the amino acids, serine and lysine, produce a pronounced animalization of the same type expected in the isolated animal half of the egg (300). The effects of these salts and organic compounds have been interpreted as meaning that the animal and vegetal parts of the egg have different types of metabolisms which respond, as would be expected, differently to inhibitors and stimulators of metabolism.

Direct proof of a metabolic difference has been difficult to establish, but it now seems quite clear that there are differences, which become particularly prominent just before the onset of gastrulation. Boveri (65) had early postulated the existence of stratification along the animal-vegetal axis of the egg, in which each stratum had a different developmental significance or fate. This he maintained was in the nature of a gradient of reactions from one pole to the opposite one (66). Some evidence for the existence of such a gradient was obtained by Child (106), who showed that in certain developmental stages up to the late blastula there is a gradient of reduction capacity. The most rapid reducing region is at the animal pole and the least rapid at the vegetal. Lindahl and Holter (378) were, however, unable to detect any evidence of this gradient by using the Cartesian diver technique of determining the respiration of isolated animal and vegetal halves of eggs. Both halves were found to have identical respiratory rates. The same was found to be true for the

dipeptidase activity of the two halves (378). Undoubtedly more refined methods are needed to detect any differences in metabolism which may exist between the two parts of the egg. One method of attack developed by Gustafson and Lenicque (241) shows great promise in establishing such a difference in the late blastula. These workers have shown by use of the phase contrast microscope and vital stains that a rapid increase of mitochondria in the sea urchin

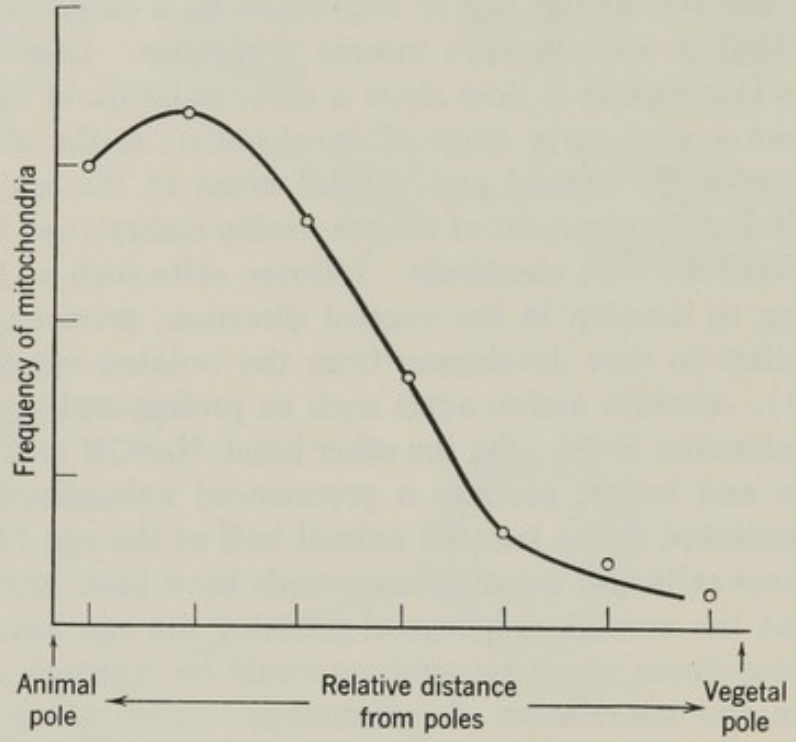


Fig. 105. Mitochondrial frequency distributions at different levels in the sea urchin blastula. These frequency distributions are for blastulas 18 hours after fertilization. After Gustafson and Lenicque (241).

during development starts at the end of the blastula stage in an intermediate stage known as the mesenchyme blastula. At this time in development the mitochondria begin to increase rapidly in number and there develops a gradient of mitochondrial distribution with far more mitochondria present in the animal than in the vegetal regions (Fig. 105). Since the mitochondria are important seats of oxidative metabolism, this observation in itself establishes a quantitative metabolic difference between the animal and vegetal areas.

It is interesting to compare the distribution of mitochondria with the data on chemical changes which occur during the early stages of sea urchin development. Some of these data are collected together in Table 51. They show quite clearly that there is not much change

Table 51. Some Estimates of Changes in Chemical Composition and Activity of Early Sea Urchin Embryos During Development *

	Nature of Change if Any	Stage at Which Change Occurs	Effect of Li^+
	Nitrogenous Constituents		
Total nitrogen	No change	—	—
Ammonia	3-fold increase	Mesenchyme blastula	Increase retarded
Amino acids	Changes in some	Mesenchyme blastula	Retardation of changes
	Enzyme Activity and Concentration		
Apyrase	2-fold increase	Mesenchyme blastula	Increase retarded
Succinic dehydrogenase	More than 5-fold increase	Mesenchyme blastula	Increase retarded
Malic dehydrogenase	3-fold increase	Mesenchyme blastula	—
Glutaminase	10-fold increase	Gastrulation	Increase retarded
Cathepsin II	Weak or zero until change	Mesenchyme blastula	Increase retarded
Alkaline phosphatase	Increase	Mesenchyme blastula	Slight retardation
Pyrophosphatase	No change	—	No effect on activity
Phosphomonoesterase	No change	—	No effect on activity
Aldolase	No change	—	—
Adenosine deaminase	No change	—	No effect on activity
Phenylsulfatase	No change	—	—
Cytochrome	Increase and subsequent decrease	Increase after fertilization, decrease in blastula	—
Catalase	Decrease	Mesenchyme blastula	Decrease abetted
	Respiration and Enzyme Complexes		
Respiratory rate	5-fold increase	Fertilization, and then again at mesenchyme blastula	Checks increase
Number of mitochondria	Definite increase	Mesenchyme blastula	Retards increase

* Data from 240, 241, 325, 58.

in chemical activity, for those things measured, until the mesenchyme blastula stage when a number of different enzymes increase in activity, if not in amount. It will be noted that coincident with the increase in activity of these enzymes there is also an increase in respiration and the number of mitochondria. Certain of the enzymes such as succinic dehydrogenase which increase in activity are known to be important elements in the mitochondria of other animals and because of the coincidence noted here may well be in the sea urchin.

The effect of Li ion previously referred to as an agent causing vegetalization is also of considerable interest in connection with the activities listed in Table 51. In all those cases in which there is an increase in activity at the time of formation of the mesenchyme

blastula, there is a decided retarding effect of Li^+. This is true of the increase in the mitochondrial frequency as well as the increase in enzyme and respiratory activity. Lithium not only retards the increase of mitochondria, but, what seems to be more important according to Gustafson and Lenicque (241), causes a reduction in the gradient of mitochondrial frequency between the animal and vegetal regions. These results would make it appear probable that one of the major factors causing the difference between the animal and vegetal regions is the mitochondrial gradient with its resultant effect on metabolism. Any disturbance of this gradient, as for example by the action of Li^+, causes a reduction in the difference in metabolism between the two parts of the embryo, and leads finally to abnormal development.

Whether the mitochondrial gradient existing in the sea urchin egg is the cause or the effect of the animal area developing ectodermal, and the vegetal primarily endodermal derivatives, is impossible to decide on the basis of the present evidence. That there is some relationship does seem certain. If the observed gradient is assumed to be the cause, for purposes of discussion, the logical question then arises as to what causes the appearance of the gradient. There are no experimental data to give a definite answer to this question. Gustafson and Lenicque (241), drawing upon the hypothesis of Runnström (523*a*) that there are two opposing gradients of activity in the sea urchin eggs, one with its greatest intensity at the animal pole and the other at the vegetal pole, have further hypothesized that these gradients may be the reason for the existence of the mitochondrial gradient. These workers postulate that mitochondrial production and protein synthesis is the chief activity of the animal side whereas the vegetal region is chiefly engaged in protein degradation, pigment synthesis, and sulfate esterification. These two comprise competitive types of metabolic patterns. The weakening of one causes a strengthening of the other. Hence any reduction in mitochondrial number by Li^+, etc., causes a strengthening of the vegetal type of metabolism and excessive endoderm formation.

The problem of how these primary differences arise in the egg is of great importance, but there is scarcely any information about it. Conceivably gravity acting upon particles of different specific gravity could cause a stratification which forms the basis for the more drastic differentiation which follows. Costello (118) has suggested that diffusion effects may be of great significance, and has developed an elaborate hypothesis based in part on the assumption that the egg

surface may have areas of different permeability characteristics. This is not unlikely, considering the differences known to exist in the cortex of some eggs. Such differences in permeability with respect to intake and outgo of various food and excretory materials would be expected to set up a series of diffusion gradients within the egg which would be expected to have considerable effect on the course of metabolism within the different regions of the egg.

Whatever is the immediate cause of the initial organization of the egg cytoplasm, it must be recognized that this organization occurs in the presence and undoubtedly under the control of genes. The cytoplasm of the zygote while not initially a product of the genome within it, must be considered as having been produced primarily under the control of the maternal genotype at the time the egg is formed in the ovary.

The demonstrated organization of the egg cytoplasm may be taken as the first significant step toward the later differentiation of cells derived from it, and it therefore plays an important role in the mechanism of development and the determination of the phenotype.

Chemical Events Accompanying or Preceding Morphological Changes

It will be recalled that the determinations of enzymatic activity of embryonic chick tissue showed definite changes in some enzymes as development proceeded (p. 349). Furthermore, it will be noted from Table 51 that a number of enzymes increase in activity and presumably in amount at a time just preceding the onset of gastrulation in the sea urchin. Such changes in metabolic activity are to be expected, if it is assumed that chemical differentiation must accompany morphological differentiation. However, some of the facts known about chemical changes occurring in this connection are of significance in interpreting some of the further aspects of development.

The considerable work done by Brachet and others, reviewed by Brachet (67), on the chemical events during early development of the Amphibia will serve to illustrate two important points. (1) Chemical change occurring during development may be initiated quite suddenly and accompany or even precede very important phases of development, such as gastrulation. (2) Chemical changes may not always be general over the whole embryo but may be quite localized, leading to further heterogeneity than may have first been present in the egg. As in the sea urchin (Table 51), there is no demonstrably significant chemical change in metabolic activity in the frog during

the cleavage and blastula stages immediately following fertilization. This is reflected in a relatively constant oxygen uptake and a respiratory quotient of about 0.60 to 0.65. During this early phase the embryo is capable of cleaving anaerobically, and KCN has no effect at concentrations which would be immediately toxic to the embryo in later stages of its development. With the onset of gastrulation, however, an immediate change occurs. The respiratory quotient goes up to 0.95 to 1.0, a KCN sensitivity develops, and the absence of free oxygen causes a definite inhibition of further development. A blastula, for example, will not gastrulate in the absence of oxygen. Accompanying this change in respiration is a change in carbohydrate utilization. Prior to gastrulation the carbohydrate reserve remains fairly constant in the form of glycogen, but with the onset of gastrulation glycogen begins to disappear.

Brachet (67) arrived at the reasonable conclusion from these data (and other supporting data not given here) that, during cleavage and blastulation, oxidation is incomplete, and the embryo draws upon its energy reserves in the form of ATP and phosphocreatine, both of which can be demonstrated to be present. A terminal oxidase system in the form of flavoprotein may function in the presence of oxygen, but the cyanide insensitivity would indicate that a cytochrome system is not functioning. With the onset of gastrulation, the metabolic machinery of respiration changes drastically. Glycolysis commences, a cytochrome system presumably appears, and in general respiration assumes its usual aspect.

It is certainly significant that in both the sea urchin (see Table 51) and in the frog there is a definite change in metabolism, especially as regards respiration at the time of gastrulation. This change is not gradual, but very rapid, occurring in the frog in a space of a few hours. A similarly abrupt change in respiration occurs in the development of insects just after the onset of pupation.

The metabolic localization in the egg is continued and certainly enhanced in the embryo. An example has already been given of the distribution of mitochondria in the sea urchin. In the frog Brachet (67, 70) has shown that the distribution of sulfhydryl proteins in the egg is unequal. There is a gradient of distribution from the animal to the vegetal poles with the highest concentrations at the animal pole. In the gastrula the highest concentration is in the dorsal lip of the blastopore, and as development continues on into the neurula stage the main fraction of this protein is found in the neural plate, particularly in the anterior end. Ribonucleic acid or ribonucleopro-

tein is distributed similarly to the sulfhydryl proteins according to Brachet (67, 70). Glycogen is most heavily concentrated in the blastula and early gastrula of the frog in the animal (or dorsal) part of the embryo (267). With the onset of gastrulation the glycogen in the region of the dorsal lip of the blastopore disappears most rapidly whereas that in the other regions do so much more slowly (Fig. 106)

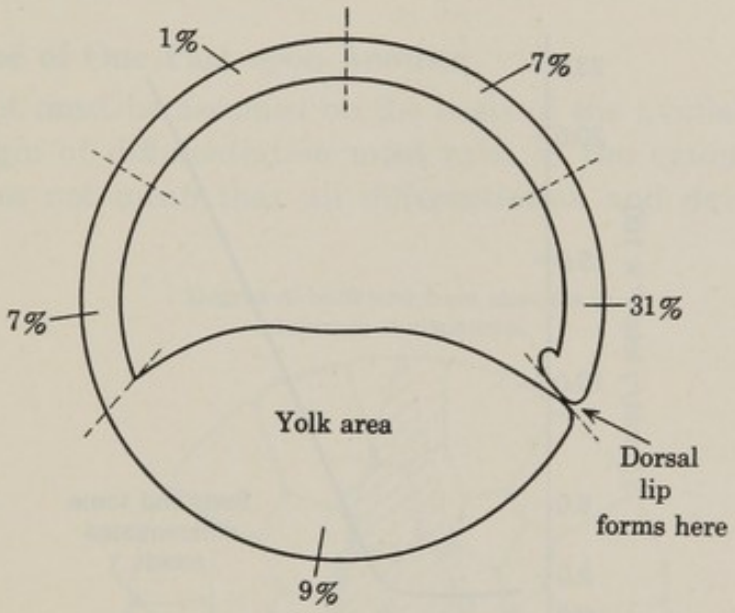


Fig. 106. Early frog gastrula in section showing the approximate per cent decrease of glycogen content of the various parts of the gastrula from the time of onset of gastrulation to its end. After Heatley and Lindahl (268).

(268). Correlated with this glycolysis is an increase in oxygen consumption in the dorsal region of the embryo, specifically in the region of the dorsal lip, at the time of gastrulation.

The literature of biochemical embryology is replete with examples of localization of specific types of compounds, thus indicating areas with differences in metabolism. Gradients are generally associated with these localizations, for the distribution of most compounds is not all or none. Rather there are centers of concentration with gradients of reduced amount outward from the center. Gradients have been noted in the developing embryos of nearly all animals. They have been investigated from different points of view—some merely from the descriptive chemical—others from the physiological. Child (106) has analyzed the physiology of development of many organisms from the standpoint of his gradient theory which in essence simply states that there are various centers of activity set up at different points in the developing embryo. Initially there is one—the animal vegetal, then

a second, etc.—each following the other until the embryo is comprised of what might be assumed to be a large number of different metabolic centers of activity. The activities of some of these may be synergistic, others may compete for common substrates, and still others may inhibit one another because of the products they produce.

Further and very significant examples of chemical changes during development can be found in the many experiments in regeneration.

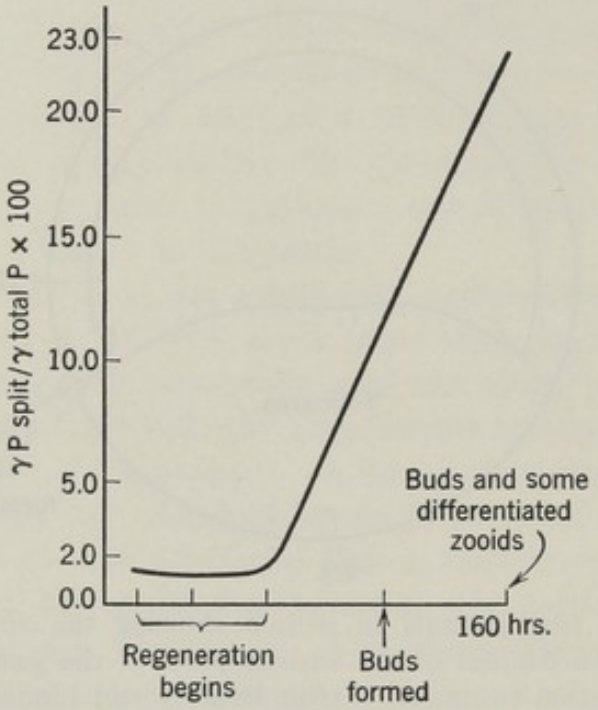


Fig. 107. Increase in adenosine triphosphatase (apyrase) activity in the ascidian *Symplegma*. *Symplegma* is a colonial ascidian which forms zooids from buds off the stolon, or central stem of the colony. When the stolon begins to regenerate a zooid, the ATPase activity increases at a high rate. After Jaeger and Barth (314).

Regeneration must be considered as an aspect of development as has been emphasized repeatedly by Child (106). It is noteworthy that, as a part of regeneration, chemical changes occur which reflect the same changes that original parts of the organism underwent during embryogeny.

Some examples of chemical changes accompanying regeneration or additional growth associated with differentiation may be found among the observations on certain coelenterates and ascidians. In the coelenterate *Obelia geniculata,* those areas of the colony which are in active proliferation show heavy concentrations of free sulfhydryl groups in the endoderm (104). Other areas of the endoderm show only diffuse sulfhydryl activity. In the ascidian *Symplegma,* a colo-

nial form which has zooids (adults) budding off a stolon, Jaeger and Barth (314) have shown that, whereas the stolons have a very low activity of apyrase (adenosine triphosphatase) those stolons or fragments of regenerating stolons about to produce zooids have a very high activity. Hence development from a relatively undifferentiated mass of cells making up the stolon into a differentiated stolon is accompanied by a many fold increase in apyrase (Fig. 107).

The Influence of One Part upon Another

Although it must be assumed on the basis of the available evidence that the origin of differentiation must exist in the cytoplasm of the egg, this does not mean that all differentiation and development is

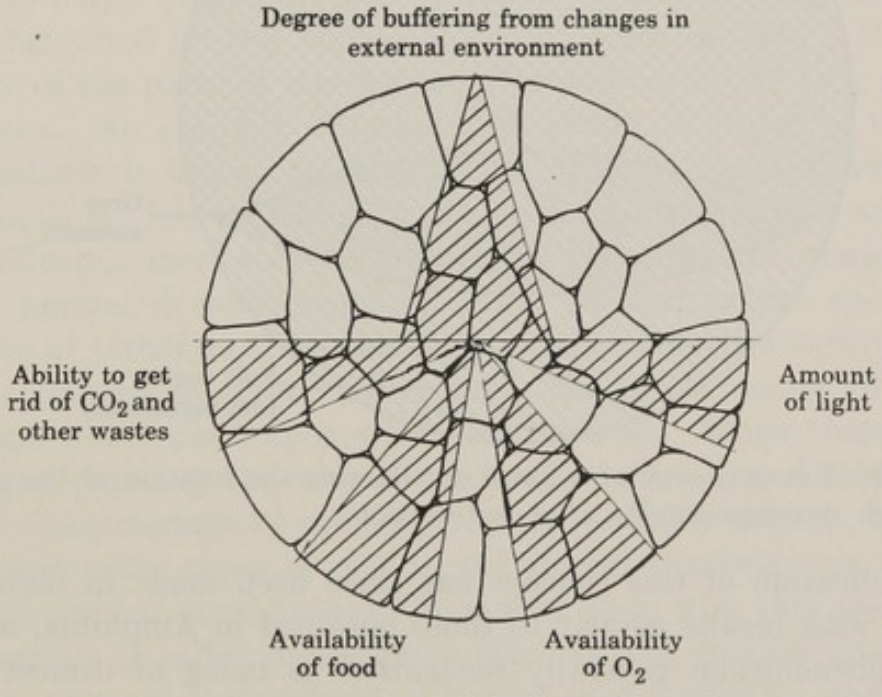


Fig. 108. Diagram to illustrate some of the internal environmental differences inside a ball of cells. The shaded triangular areas are intended to show that some factors increase in intensity, and some decrease in intensity, from the surface to the center of the cellular mass.

solely the result of a cytoplasmic sorting out or segregation. The intrinsic factors which make the cells different during cleavage set the stage for the next phase of development in which the differentiated cells begin to affect one another. Upon the primary differentiation resulting from segregation of capacities during cleavage there must be superimposed a second phase of differentiation arising from new environmental circumstances being created around each cell or cell layer,

owing to differences in its neighbors. The extent to which the environment around each cell may be different may be readily appreciated by considering the consequences of the formation of a ball of cells (see Fig. 108).

The critical proof of the effect of one type of cell on another was advanced by Spemann as a result of his experiments on the amphibian embryo which culminated in the concept of the "organizer." The gen-

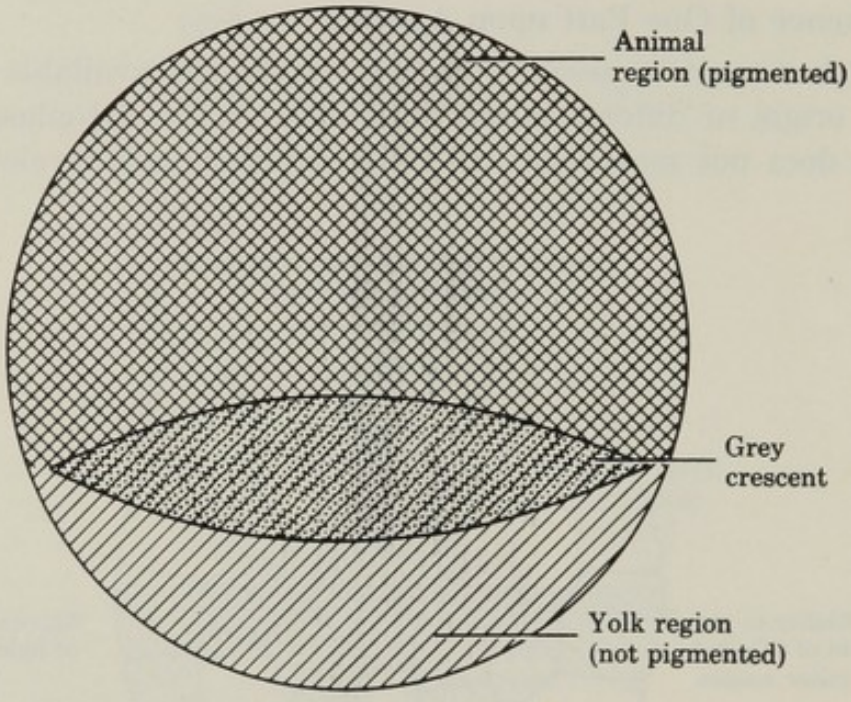


Fig. 109. External view of a frog's egg showing the location of the gray crescent which develops shortly after fertilization.

eral application of this concept has since been made in many other animals with results similar to those obtained in Amphibia, and it is now a phenomenon generally recognized as being of utmost importance in the development of all vertebrate animals. It is probably also of significance in development of most invertebrate animals with the exception of those which show strict determinate development from mosaic-type eggs.

The best examples of these influences are to be found in the Amphibia, which have a regulative type of development and an egg which can be kept under continuous observation from the very beginning of the embryonic development immediately following fertilization. This egg shows both regulative and mosaic properties similar to those already indicated for the sea urchin. Immediately after fertilization there appears a crescentic-shaped gray area at a point near the fusion of the animal and yolk areas of the egg (Fig. 109). It is mainly con-

fined to the cortex of the egg and it is called, because of its appearance, the gray crescent. It is an area of utmost significance in the developing egg, as indicated by (1) it being the location of the future dorsal lip of the blastopore and (2) the fact that the egg cannot develop normally without it. As in the sea urchin, bisecting the frog egg in a plane through the animal and vegetal poles gives two perfect larvae. However, the plane in the frog must also bisect or at least divide the gray crescent so that there is gray crescent material in both. Failure to do so results in only one larva while the half without the gray crescent undergoes a few cleavages and stops further development. This demonstrates the mosaic qualities present in the frog's egg. Beyond this it is regulative to a marked degree as demonstrated in the following discussion and examples.

A "fate map" (Fig. 110) can be made for a frog's egg similar to those constructed for the Mollusca and Annelida (p. 355). However, the fates of the parts of the egg are *presumptive* in the frog and not *determined*. An example will make this clear. A region of the very early gastrula is known to go into the formation of the adult eye. That this is its fate can be demonstrated by marking the area with a non-diffusible inert substance and then following the marked area through normal development to its new location in the eye region. If a piece of ectoderm from this area is excised and transplanted into an older embryo as shown in Fig. 111, the future course of its development will depend upon where it is placed. When transplanted to the head region of the host it will form eye, brain, and mesodermal material characteristic of the head region. In other regions, however, it will form other types of organs and tissues characteristic of those regions in normal development. It is now clear why this area in the early gastrula is called *presumptive* eye. It will develop into an eye only in the proper environment or spatial relationship to other organs and tissues. If put into a "neutral" environment, saline for example, it will continue to exist only as undifferentiated ectoderm. As presumptive eye tissue it is competent to react in a number of different ways and hence demonstrates a regulative type of development.

When the gastrula is allowed to continue to develop to the neurula stage in which the nerve cord begins to form, it is found that what formerly was merely presumptive eye now becomes *determined* eye. Transplanting this same area to an older embryo results only in the formation of an eye, no matter in what area introduced (Fig. 111).

Two very important conclusions can be derived from these experimental observations, which incidentally have been repeated in other

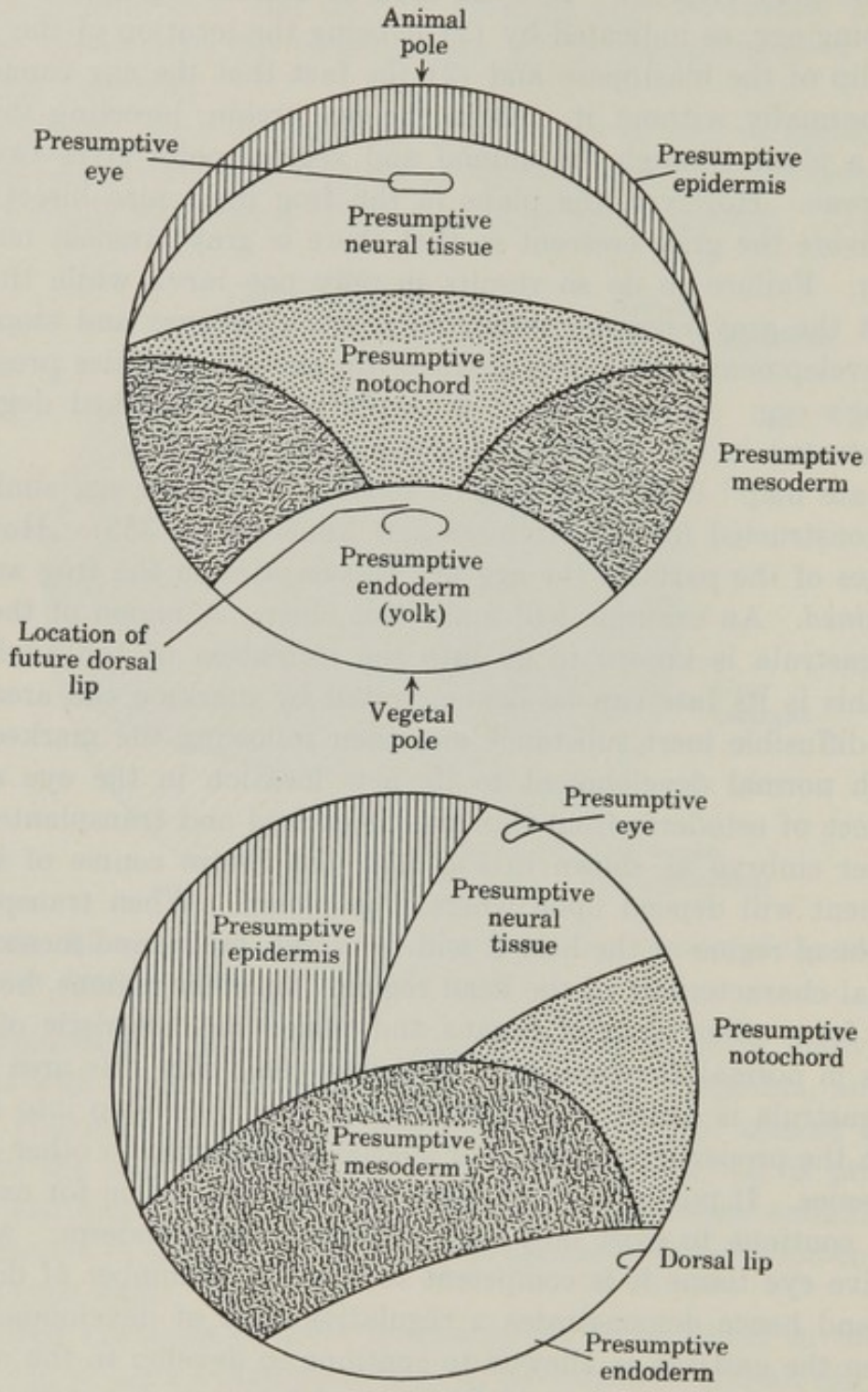


Fig. 110. Two views of the late blastula of the frog, showing the presumptive values of the various parts of the surface.

types of vertebrates, as well as with different presumptive areas of the early frog embryo. (1) The fates of the parts of the early embryo (for example gastrula in frog) are determined to a large extent by their location relative to other parts. They are, in other words,

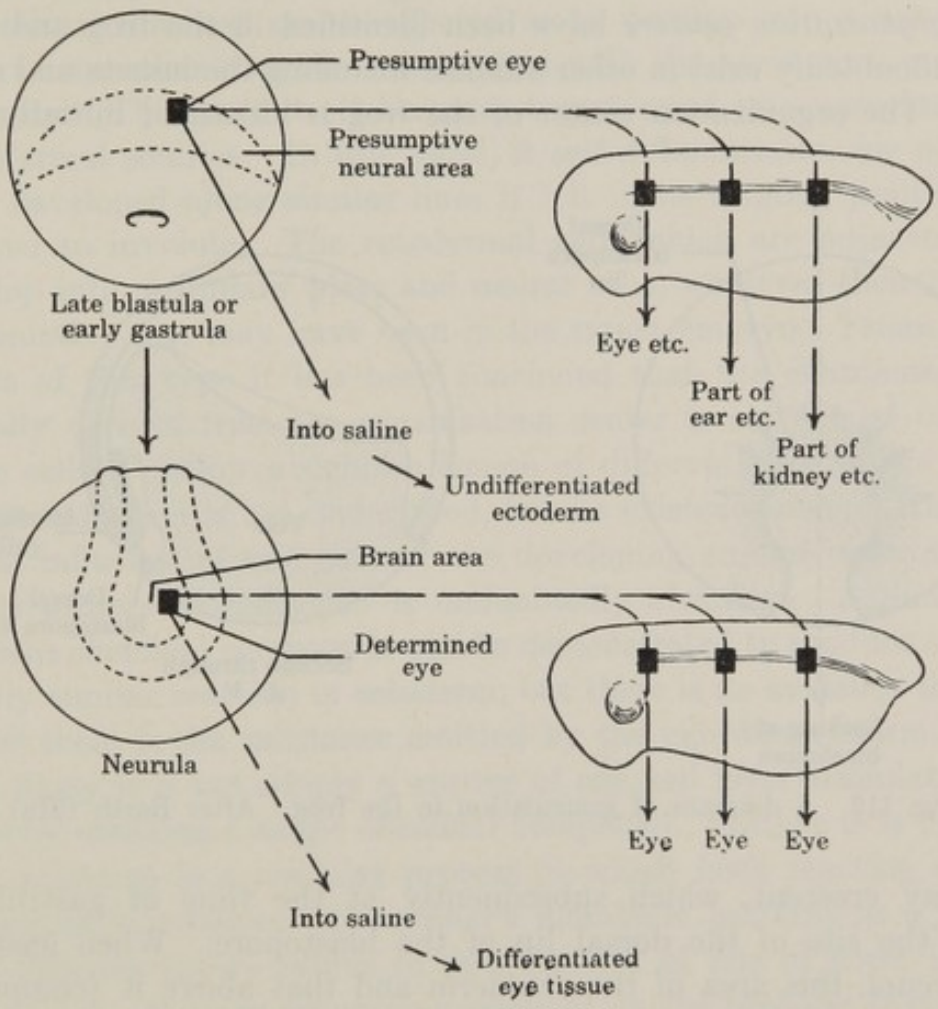


Fig. 111. A diagram of a series of transplantation experiments in the early frog embryo. The presumptive eye area of a late blastula can develop either into an eye or into entirely unrelated parts, depending upon the region in the host to which it is transplanted. If transferred to saline it will not differentiate. If, however, the eye area is removed shortly after gastrulation is completed, its fate is determined, and it will develop into eye tissue regardless of the environment in which it is placed. After Barth (21*a*).

competent to develop in many directions. (2) The period of competence or flexibility is not indefinite. There arrives a time during development (neurula stage in case of eye of frog) when the cells, because of their prolonged association with a specific type of surrounding cells, becomes fixed or determined and lose their competence to form more than one type of organ.

It is obvious that some area of the egg or early embryo must possess initially a pattern of determination which might be said to allow it to self-differentiate. This area in turn would be expected to influence the surrounding areas to differentiate along certain lines, and hence in a sense act as a center of organization or organizer (578). Such *organization centers* have been identified in the frog and chick and undoubtedly exist in other animals including the insects and mammals. The organization center of the frog is identified initially with

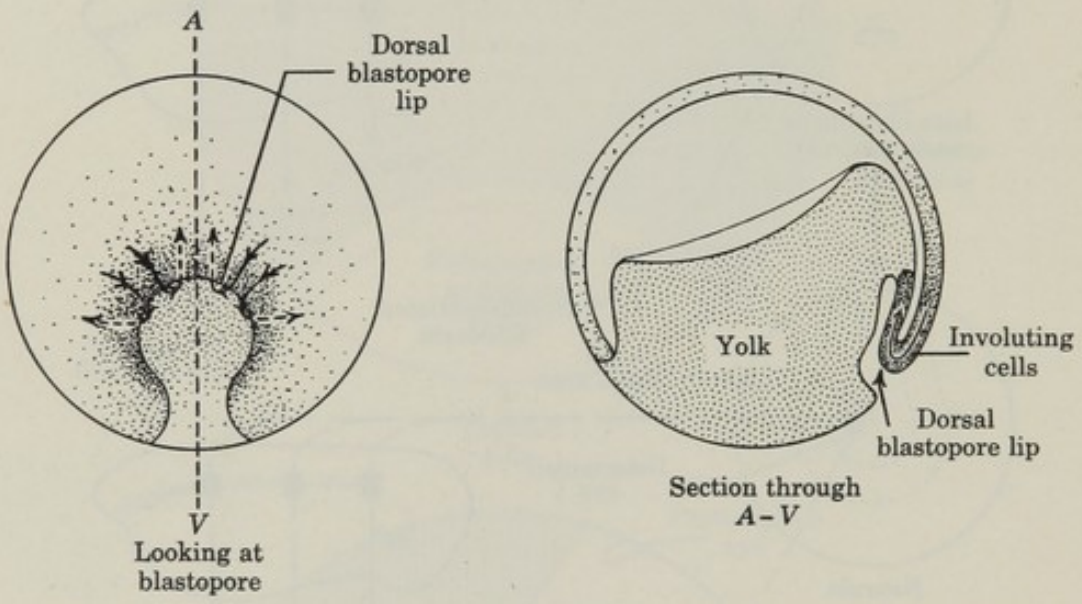


Fig. 112. A diagram of gastrulation in the frog. After Barth (21*a*).

the gray crescent, which subsequently at the time of gastrulation marks the site of the dorsal lip of the blastopore. When gastrulation occurs, this area of the ectoderm and that above it (comprising the presumptive notochord and mesoderm in Fig. 110) involutes, or folds back as shown in Fig. 112 under the ectoderm, whereas the presumptive neural tissue remains outside. The dorsal lip constitutes at this time merely a sharp corner around which the cells bend in moving from the outer surface inward. Inside the gastrula these cells become notochord and somitic mesoderm; collectively the whole invading mass is referred to as the chordamesoderm. The ectoderm immediately above the notochordal material develops into a neural tube and brain parallel to the notochord. The ectodermal area over the somitic mesoderm on either side of the notochord is destined to become epidermis and organs of epidermal origin.

This normal course of developmental events in the frog is dependent first upon the appearance of the dorsal blastopore lip in

the gray crescent area. If this organization center is removed, gastrulation cannot proceed. Furthermore, even before involution occurs, if a piece of presumptive chordamesoderm from the organization center area is removed from the early gastrula and transplanted to another part of the same or even different embryo, the surrounding cells of the host are so affected as to develop into a nerve cord and various other structures associated with the nerve cord region of the body. The graft itself becomes transformed into notochord and mesodermal somites. In this sense, it self-differentiates, for it would have developed along similar lines if left in its original position and allowed to involute. The ectodermal cells which are adjacent to it develop into medullary plate and neural tube, whatever their original presumptive fate may have been in the intact embryo. From experiments of this type it has been concluded that the chordamesoderm initially derived from the organization center is capable of *inducing* other cells to follow a definite course of differentiation. The nature of this induction is not understood, but its existence demonstrates the actual influence of one part of the developing animal upon another.

The process of induction is undoubtedly chemical. A number of different chemical compounds can be demonstrated to produce a superficially similar reaction in ectoderm, but there is no evidence that any one of them is the substance emitted by the chordamesoderm. More than likely it is not simply a matter of one cell layer stimulating another by emitting a single chemical compound. Rather it is probable that induction is a complex process in which both reacting and inducting cells influence one another's metabolic patterns in a manner such that the future course of development of one of them becomes determined. It is an interesting sidelight on the problem that the intimate relationship between the inducting cells and the ectoderm need not be indefinite. After a period of contact the inducting cells may be removed, and the ectoderm continues development along the expected pathway to formation of nervous tissue. In this sense the chordamesoderm acts as a "trigger" to set a certain chain of events into motion. Once these events begin, the ectoderm apparently cannot be further influenced to deviate—it has, in other words, lost a degree of its competence. Differentiation is in this sense an irreversible process, but one should not place too much emphasis upon the word irreversible in this context. The experiments have yet to be done which prove that dedifferentiation is impossible.

It has already been noted that the area of the gastrula which in-

cludes the organization center has a higher respiratory rate than other regions of the egg. But it is not known what significance this high respiratory activity occupies in the role of the organization center as an inductor. Actually it is apparent that its respiration together with the ectoderm upon which it acts is higher than the sum of the activities of either alone (21).

Brachet (67) has shown that changes in concentration and distribution of nucleic acid and sulfhydryl protein occur during gastrulation in the chordamesoderm and overlying ectoderm. He has theorized (70) that this indicates active protein and ribonucleic acid metabolism in microsomes containing ribonucleic acid in the chordamesoderm, and that the synthesis has a considerable role to play in the inductive process. The presence of ribonucleic acid in high concentration in the chordamesoderm in granules identified as microsomes, and the extremely close contact of these granules with the overlying ectodermal cells, suggested to Brachet that they may pass from one cell layer to the other and hence play a role in induction. To test this hypothesis he placed a sheet of Cellophane between the chordamesoderm and ectoderm of a frog gastrula before neuralization (68). The fact that no neuralization occurs under these circumstances gives some credence to his hypothesis.

It will be noted that this concept amounts to the "infection" of one cell with microsomes of another. The implication is that the infected cells, in this example the ectodermal cells, are guided in the course of their development by the infecting particles which maintain a genetic continuity by duplication. The idea of one cell infecting another with particles during the course of development has been advanced by others besides Brachet, notably Dalcq (124), who suggested that induction may be comparable to infection. Medawar and Billingham (45) have explained the well-known spreading of pigment from pigmented skin explants into unpigmented areas of the host by the infection of the colorless surrounding cells with particles having pigment-producing capacities.

The chordamesoderm is by no means the only group of cells capable of inducing others to differentiate in a specific direction. It plays perhaps a preeminent role as an inductor since it is derived from the first area of the egg which seems to be determined, but structures induced in its presence can in turn act upon other undifferentiated tissue. Once this tissue is partially differentiated it in turn can induce

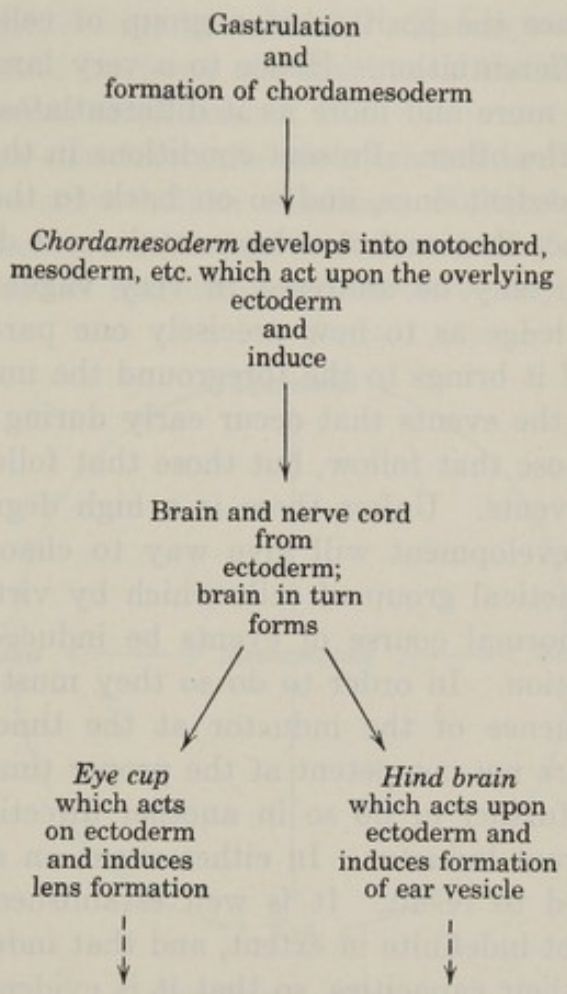


Fig. 113. An example of organizers acting in a chain.

still other undifferentiated cells. Hence one can visualize development as being in part a chain of inductions resulting from the activities of a succession of organization centers. Figure 113 gives an actual example of such a chain.

Organization and Growth

The organization of differentiated cells in space together with regulated growth gives the form and structure to the whole which results in a completed organism. The total process may be described as morphogenesis, the molding of the whole into a definite pattern, in distinction to differentiation, which is essentially a process of developing localized differences. The morphogenetic process is easily described in terms of structural change, but it is not so easily analyzed in causal terms. Even less is known about the factors involved in it than is known about differentiation. Furthermore it is quite impossible to separate aspects of morphogenesis such as organization from

differentiation, since the position of a group of cells may determine their course of differentiation. Hence to a very large extent the embryo is organized more and more as it differentiates by the influence of one part upon the other. Present conditions in the embryo are determined by antecedent ones, and so on back to the egg.

Although the fact that each developmental event depends upon preceding events can only be analyzed in very vague terms, owing to the lack of knowledge as to how precisely one part affects another, a consideration of it brings to the foreground the important factor of time. Obviously the events that occur early during development are independent of those that follow, but those that follow are dependent upon preceding events. Unless there is a high degree of synchronization, orderly development will give way to chaos. For example, consider a hypothetical group of cells which by virtue of their position will in the normal course of events be induced to differentiate in a certain direction. In order to do so they must be competent to react to the influence of the inductor at the time the inductor is active. If they are not competent at the proper time they will either not differentiate further or do so in another direction under the influence of a different inductor. In either event an abnormal embryo would be expected to result. It is well established that periods of competence are not indefinite in extent, and that inductors may themselves change in their capacities, so that it is evident that the timing must be precise to obtain precise end results (669).

A second very important factor that must be considered in connection with organization is the dynamic state of the embryo in terms of internal movements of cells and cell layers. During development the whole embryo is literally on the move internally. A considerable portion of this movement is due to differential growth, but many cells, such as the neural crest cells derived from ectoderm at the time of nerve cord formation, actually migrate from their site of origin to distant parts of the body. Neuroblasts send out processes which grow unerringly toward the organs they will enervate after becoming nerve fibers. In some animals the future germ cells originate in non-gonadal tissue and migrate to the gonads. These are a few of the many types of cell migration which characterize every developing vertebrate. To these movements of solitary cells may be added the differential growth of tissue layers which will cover some areas but not others, or the directed growth of the pronephric tubule to the cloaca to form finally the Wolffian duct.

What factors guide these movements? Unless each cell is endowed with intelligence and purpose, one must seek the answer in terms of affinities and internal environment.

It is well known that like cells are attracted to like, and that even different cell types show definite affinities. Holtfreter (287) has demonstrated that when ectoderm and endoderm cells from the early frog gastrula are explanted and combined *in vitro* they will at first tend

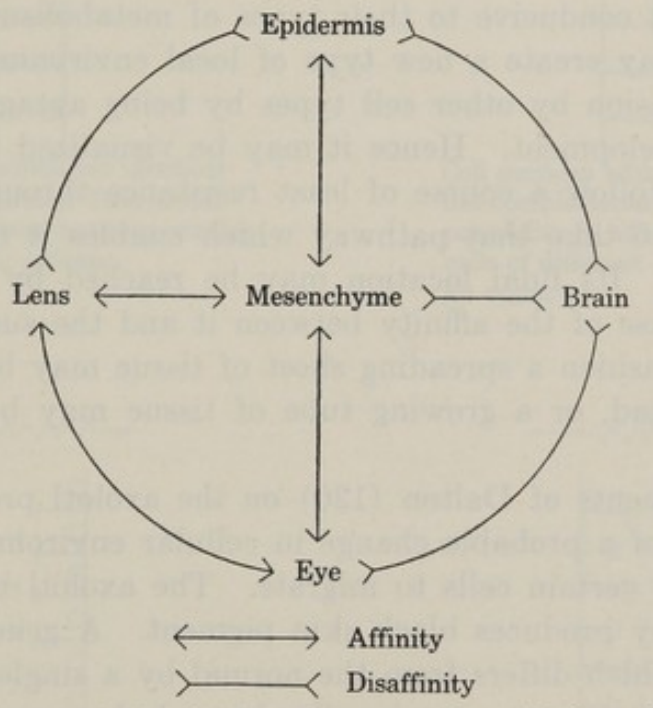


Fig. 114. Affinities and disaffinities of some embryonic frog tissues. (See text for explanation.) Data from Holtfreter (287).

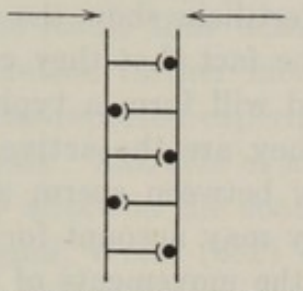
to form a common mass. In time, however, each cell type tends to congregate with its own kind, and the result is a separation into ectoderm and endoderm. Thus an initial neutrality or even affinity between unlike cells gives way in time to a strong affinity of like to like and perhaps even an antagonism between unlike ones. As differentiation proceeds, the ectodermal derivatives as an example need not, however, show affinities because of their common origin. They may indeed show definite antagonisms toward one another, and an affinity to a tissue derived from a different germ layer. Figure 114, taken from Holtfreter, indicates the affinities and disaffinities of several partially differentiated tissues of ectodermal origin. In general, mesenchyme (a mesodermal derivative) demonstrates affinities for most cell types, which makes it sort of an internal cement of the embryo.

In addition to the factor of affinity between cells of like and unlike characteristics, one must consider the internal environmental conditions within the embryo. These conditions, determined by the cells themselves, in turn determine events to follow in development as described in the foregoing. To consider the matter at the cellular or tissue level, it is quite evident that cells may congregate either because they show affinity for one another by some attractive forces between them, or they may congregate in an area because in it conditions are most conducive to their types of metabolism. Once established they may create a new type of local environment which may eliminate invasion by other cell types by being antagonistic to their continued development. Hence it may be visualized that a wandering cell may follow a course of least resistance through the embryo, being forced to take that pathway which enables it to avoid hostile environments. Its final location may be reached by chance, but it remains because of the affinity between it and the surrounding cells. In the same fashion a spreading sheet of tissue may be limited in its extent of spread, or a growing tube of tissue may be guided to its destination.

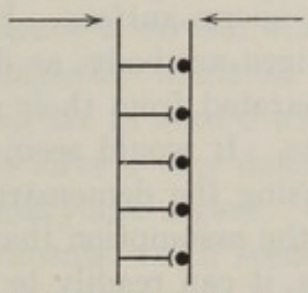
The experiments of Dalton (126) on the axolotl provide an excellent example of a probable change in cellular environment making it impossible for certain cells to migrate. The axolotl is a salamander which normally produces black skin pigment. A genetically distinct albino form which differs from the normal by a single gene is devoid of skin pigment, except, occasionally, for a dark area along the spinal column region. By means of skin grafts and the culture of tissues *in vitro* from animals of both genotypes, Dalton was able to demonstrate that the albino possesses melanophores which are capable of synthesizing melanin both *in vitro* and in the skin of the ventral side of both albino and black strains. The melanophores of the Amphibia, as in other vertebrates, arise originally in the neural crest ectoderm, an embryonic tissue which lies just dorsolateral to the embryonic spinal cord, and then migrate from there, as melanoblasts, to the various parts of the skin where they become melanophores and produce pigment. The conclusion that the albino condition is the result of an inhibition of migration of melanoblasts rather than any change in the capacity to synthesize melanin on the part of the melanophores is amply confirmed by the observation that the melanoblasts of both genotypes, albino and black, can migrate freely beneath the epidermis of the black strain, but neither can migrate in the skin of the albino.

The nature of the affinities between like and unlike cells has been

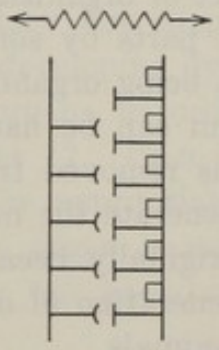
speculated upon by Weiss (682, 683), who has formulated a very inclusive and elegant hypothesis to explain it. If it is assumed that cell surfaces can exist in a variety of states with respect to the types of molecular configurations projecting from them, then it can be



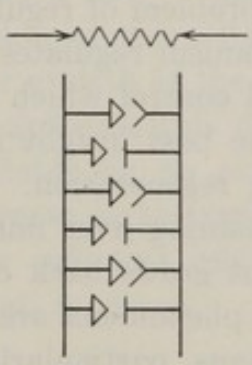
Cell surfaces which are identical and complementary. This model illustrates affinity between similar type cells as in a tissue.



Cell surfaces which are different but complementary. Illustrates possibility of affinity between cells of different tissues as in an organ.



Cell surfaces which are noncomplementary. Cells with these surfaces would show disaffinity.



Cell surfaces which are partly complementary. Cells with these surfaces may show weak affinity.

Fig. 115. Diagrams to illustrate possible types of complementary and noncomplementary surfaces. After the hypotheses Weiss (682) and Tyler (658).

readily appreciated that if two cells have surfaces with complementary configurations, as presumably exist between antigen and antibody, they will be attracted to one another. Figure 115 gives a graphic representation to explain how this concept can be applied to affinities between similar and dissimilar cells. Both attraction and antagonism can be explained on this basis, as well as degrees of affinity.

There is adequate evidence that attractive forces between cells may be interpreted by the application of immunological theory. The at-

traction between the sea urchin sperm and egg can, for example, be explained as the result of the complementariness of the surfaces of these cells (658). The egg contains a protein, fertilizin, in its gelatinous coat which can be separated from the egg and shown to cause an agglutination of sperm by reacting with the protein, antifertilizin, on the sperm surface. Fertilizin and antifertilizin show the relation of antigen-antibody, as demonstrated by the fact that they can both be separated from their respective cells and will form a typical precipitate. It would seem clear then that they are the active factors in causing the demonstrated strong affinity between sperm and egg.

On the assumption that surface specificity may account for cell affinities, it can readily be appreciated that the movements of isolated cells, the growth of cell processes, and the growth of tubes and sheets of tissue may be guided in predetermined pathways by "tracks" of complementary surfaces. This factor may operate in addition to and in conjunction with that of following the path of least resistance as discussed in the previous paragraphs.

The problem of regulated growth is an aspect of organization, since the organism regulates the growth of its own parts by some sort of internal control which is a direct result of its being organized. Perhaps the best insight into the general problem can be had by considering regeneration. If a piece of a liver is removed from a rat, the remaining liver immediately begins to regenerate the missing tissues, but grows back only as much as had originally been removed. Similar phenomena are to be noted in the regeneration of other parts and organs, particularly in the invertebrate animals.

What type of control exists to determine that an organ will grow to a certain size and then cease growth? Obviously, there are many factors which may be involved. The volume-surface relationship, so often implicated as the cause of cell division, may be one factor; the competition for available substrate materials for nutrition, another. Finally, there must be considered the direct influence of one part upon another by the action of hormones, the nervous system, etc. The net effect of these factors is a dynamic equilibrium giving the relatively constant internal milieu of the adult so much emphasized by Claude Bernard. This equilibrium, which is easily visualized as being the result of the interplay of parts manifested as self-regulation or homeostasis, must be considered as a factor in development. The growth of a part will naturally depend to a large extent upon the materials available which may be in limited supply, and in part upon the action of hormones and the nervous system in an animal.

Auto-inhibition has come to be recognized as an additional possible factor in the regulation of growth. The growth of an organ to a particular size can be readily explained if as a result of its metabolic activity it produces substances which inhibit specifically its own metabolism. The larger the organ grows, the more inhibitor is produced, with the result that when the inhibitor reaches a certain threshold concentration further increase in size in completely hindered. Rose (521) has reported experimental results which are in accord with this hypothesis. Extracts of adult chicken organs have a very definite inhibitory effect on the continued growth of embryonic organs. On the other hand, Weiss (682) has reported experiments which seem to be directly contradictory to the above results. Fragments of liver from 6-day-old chick embryos when implanted in the area vasculosa of 4-day-old chicks demonstrate a definite stimulatory effect on the growth of the host liver. The effect is fairly specific, although there is a slight general reduction in growth on the part of the rest of the embryo. Actually, of course, these two sets of data need not be considered contradictory but supplementary. The addition of more liver to a partially developing liver simply permits faster growth at the expense of other organs, according to the principle of autocatalysis discussed in Chapter 12. Once the production of inhibitor by an organ has reached the appropriate level, its further increase is restricted. An embryonic organ is not capable of producing sufficient inhibitor to effect this, but an adult organ produces enough to do so.

2. The Role of the Genotype

The most direct method of analyzing the role of the genotype in development is to determine the effect of a mutant gene by comparison with the effect of its normal allele. This method merely pushes back the analysis of phenotypic difference to whatever stage in development the mutant individual begins to show differences from the standard. As in any study which presumes to analyze gene action such an approach does not necessarily reveal what each gene does in development, but only what the two by comparison do differently.

Effects of Gene Mutation on Development

Developmental studies in which mutant strains have been compared to the wild type have been made on a great number of different organisms, but primarily on chickens (254, 309, 338, 339, 340), mam-

mals (203, 204, 237, 238, 239), and *Drosophila* (245, 494). A few examples from among the many observations made by the workers in this field will serve to illustrate the methods of analysis and the type of results obtained.

Mutant characters expressed as lethals at some stage in development have been extensively studied in *Drosophila.* By comparing the effects of a series of homozygous recessive X chromosome deficiencies in *D. melanogaster* on the embryonic, larval development within the egg, Poulson (494) was able to show that different lethal effects result from deficiencies of different parts of the X chromosome. The complete absence of the X chromosome results in disorganization of the egg during early cleavage and the death of the egg before a blastoderm is formed. Homozygous deficiencies of small segments of the X are also lethal, but, in general, embryonic development proceeds to a later stage before death ensues. A group of small deficiencies in the region of the white locus all produce essentially the same type of embryonic disturbance between the 12th and 16th hour of development. Organs and parts of mesodermal and endodermal origin show highly abnormal development, whereas the ectodermal derivatives develop apparently undisturbed until death caused by the abnormal development of other parts ensues. Deficiencies including the facet locus or a region just adjacent to it cause a lethal effect in less than 12 hours. All three germ layers of the embryo are definitely disturbed in their development.

Lethality due to mutation can occur at any stage in the life of *Drosophila,* depending on the type of lethal mutation. Table 52 gives a short list of mutant genes with lethal effects and a description of the abnormalities which presumably are directly related to the death of the organism. It will be noted that for each mutant gene example death occurs at a different time in development and from different causes, or at least accompanied by different symptoms.

Observations made on other animal forms have given similar results, whether they have been studies of lethal phenotypes or viable morphological mutants. The mutant gene effect as compared to the normal is to cause an upset of the normal course of events at a specific period of development which ends either in death or an abnormal phenotype. As far as can be determined from the available data, the mutation of each gene has its own characteristic effects on the pattern of development.

A gene change generally results in more than one observable change in phenotype. Just how many changes are observed is a function of

Table 52. Postembryonic Lethals of *Drosophila* *

Gene and Symbol	Stage of Death	Description of Abnormality
Cy (*Curly*)	At time of hatching from egg	Inability of larvae to continue further development after completion of embryonic development
B^{263-20}	First instar	Defective ring gland
1(2)*me* (*meander*)	Third instar	Inability to metabolize food in second half of larval life; general slowdown of growth; does not pupate; respiration extremely low in second half of larval life
l(2)*gl* (*lethal giant larva*)	End of third instar at time of pupation	"Pseudopupae" formed; larva ceases further development within puparium; main imaginal discs and male germ cells degenerate before death of larva
l(3)*tr* (*translucida*)	Early or late pupal life	Larvae show abnormal signs early as first instar, small fat bodies, swollen with excessive hemolymph and quite transparent; after pupation imaginal differentiation becomes almost completely abnormal
crc (*cryptocephal*)	Late pupal life	Head does not evaginate but remains hidden in thorax
l(2)*lgt* (*leg tumors*)	Early imaginal life	Leg tumors

* Data from Hadorn (245).

the acuity of the observer. This pleiotropy has long been the subject of controversy and discussion among geneticists concerned with gene action. Developmental geneticists such as Grüneberg have clearly demonstrated that complex phenotypes caused by single gene mutations in mice or rats can frequently be accounted for as the consequence of a change in a single developmental event. For example, inherited achondroplasia in the rat (238) produces a complex syndrome in this animal which leads to early death. Among the more striking phenotypic effects are inability to suckle, high resistance in pulmonary circulation, faulty respiration and occlusion of incisors, and general arrest of development. All these abnormal conditions can be traced back to a single primary effect, an abnormal development of cartilage which first manifests itself in the early fetus. A simple "primary" cause for extremely complex end results is to be expected on the basis of our knowledge of development and metabolism. The interrelationships of the parts of the whole at all levels of organiza-

tion can only be expected to have ramifying consequences resulting from a change in any part of the system. Consider for example the disease beriberi. This disease has an extremely complex set of symptoms in its acute form which include labored breathing, rapid pulse, nausea, diarrhea, decreased urine flow, palpitations, blueness of the skin, edema, neuritis, and cardiac failure—a complex phenotype produced by a deficiency of a single vitamin, thiamine.

On the other hand, not all complex phenotypes can be related to a single initial cause in development. The careful study of the *Sd*-strain of mice by Glueckohn-Schoenheimer (202, 203) provides an interesting example of this. *Sd* (Danforth's short tail) is a dominant mutation which is lethal shortly after birth in homozygotes and semilethal in heterozygotes. Abnormalities are found in both the axial skeleton and the urogenital system; the spine and tail develop improperly; and the metanephric kidneys either fail to develop at all (homozygotes) or are quite abnormal (heterozygotes). A detailed study of the early stages of development failed to reveal any single causative factor which manifested itself morphologically to connect abnormalities in these two systems. Both systems are of mesodermal origin, but other mesodermal derivatives do not appear to be affected by the mutation. Other examples of extreme pleiotropy which are difficult to resolve with a one-primary-function hypothesis for the gene may be found among the mutations that affect coat color in the mammals. Generally, these have effects in addition to changes in pigmentation. The gray lethal mutant of the mouse apparently blocks the production of yellow pigment in the hair and causes gross abnormalities of the skeletal system (237). In the same animal, the yellow coat color mutation, A^y, one of the alleles of the agouti series, is lethal when homozygous but produces viable offspring with yellow coat color when heterozygous (517). It is difficult in this example and the preceding one to relate the mere absence or presence of a melanin pigment with extensive internal abnormalities. The A^y gene homozygous actually results in cessation of development in the blastocyst (blastula) stage.

It is quite impossible at the present time to resolve the question of whether each gene is uni- or multifunctional in its primary action. Grüneberg (238) has with a number of other geneticists decided on the basis of developmental studies in mammals that no example of pleiotropy is "genuine." They are all "spurious," because it should always be possible to show that a single developmental change can cause the apparently unrelated phenotypic changes resulting from the

mutation of a single gene. The question will only be answered with a further knowledge of gene action. Or it may transpire that the question, as such, has little meaning. For the present there are no compelling reasons to take a firm stand for either side of the question.

It is not unusual to find, in investigating the developmental mechanics of mutant phenotypes, that certain organs or tissues show normal development from a very early stage in embryogeny, then suddenly begin to develop abnormally. Frequently a regression or perhaps dedifferentiation sets in, and the affected tissue may all but disappear. This phenomenon is illustrated in the inherited recessive rumpless condition found in chickens (726) which results in the incomplete development of the tail axial skeleton. The tail rudiment, including both the mesodermal and neural tissue elements, develops normally to at least the middle of the fourth day. At this time the neural tissue forming the nerve cord of the future tail begins to degenerate and disappear, accompanied to some extent by the degeneration of notochordal material. In contrast to this condition in recessive rumpless, a superficially similar condition, dominant rumpless, demonstrates abnormalities of the tail bud at the time of its first appearance in embryogeny.

A degeneration similar to recessive rumpless in the fowl is found in the *Sd* mutant of the mouse. In this mutant the tail develops normally up to the age of 10 days after fertilization. At this juncture all parts of the tail begin to show signs of degeneration, and by the twelfth day the neural tube, gut notochord, and somite components of the tail have disappeared.

Degenerative phenomena are not unknown in normal development, as for example the breakdown of larval tissue in insect pupae, and probably therefore should not be thought strange in abnormal conditions. The dramatic shift, however, in the direction of development backward as the result of a single gene change, is of considerable interest, nonetheless. It may be a manifestation of the failure of the proper inductor to develop at the right time in some cases; and in others it may be due to improper metabolic conditions in the locality in which the degenerating tissue finds itself. On the other hand, the determining factor may be intrinsic and quite independent of external influences. A means of approaching this general problem has been found in transplantation experiments discussed in the following section.

Transplants between Different Genotypes

A considerable amount of attention has been given to transplanting a piece of tissue of one genotype into a host of another with the object of determining the reaction of the transplant. In general, the results have been quite simple; the transplant develops autonomously according to its own genotype as if it had not been transplanted. The few exceptions which have demonstrated heteronomous development have, however, been most instructive in demonstrating the interrelations which exist in development by showing that some parts of an embryo may be affected only indirectly by a mutant gene which presumably exists in all cells.

The heteronomous development of transplants has already been illustrated in the development of eye color in *Drosophila* (p. 216). It will be recalled that vermilion and cinnabar eye anlage when planted in a larva of normal genotype responded by producing brown pigment. The underlying cause for failure to develop pigmentation was discovered to be a metabolic upset in the mutants, resulting in their inability to form the intermediates for eye pigment production. The intermediates happen, in this example, to be readily diffusible through the bodies of the flies, and hence are supplied by the normal host—an effect on the phenotype identical in many respects to the induction of growth in a nutritional mutant of *Neurospora* on the addition to the medium of a required compound. It is noteworthy, however, in the example of *Drosophila,* that, of 30-odd mutant eye color anlage of different genotype tested by transplantation, only these two genotypes have shown a heteronomous response (29, 30, 170*a*). Precisely why vermilion and cinnabar are heteronomous and the others not is unknown, but it is reasonable to assume that factors such as permeability, diffusibility, and cell or tissue organization may well be involved.

The developmental aspects of the inherited *Creeper* condition in the fowl have been studied to some advantage by implanting the embryonic parts of mutant fowl into the chorioallantoic membrane (extra-embryonic membrane with a respiratory and excretory function), coelom, or eye regions of normal hosts. The *Creeper* condition is caused by a dominant gene which is lethal homozygous. The homozygous embryos die in the third or fourth day of incubation, presumably because of an aberrant yolk sac circulation. Occasionally a "breakthrough" occurs, and a few individuals develop almost until the end of the incubation period. (See Fig. 116.) These individuals show

extreme malformation of bone and cartilage structures (254). The eyes are also affected. These are smaller than normal and afflicted with colomba (339). The heterozygotes are viable but have extremely short and bent legs resulting from the abnormal development of cartilage preceding bone formation (339).

By means of growing tissue explants of 60-hour homozygous *Creeper* embryos in tissue culture and in the chorioallantoic membranes of

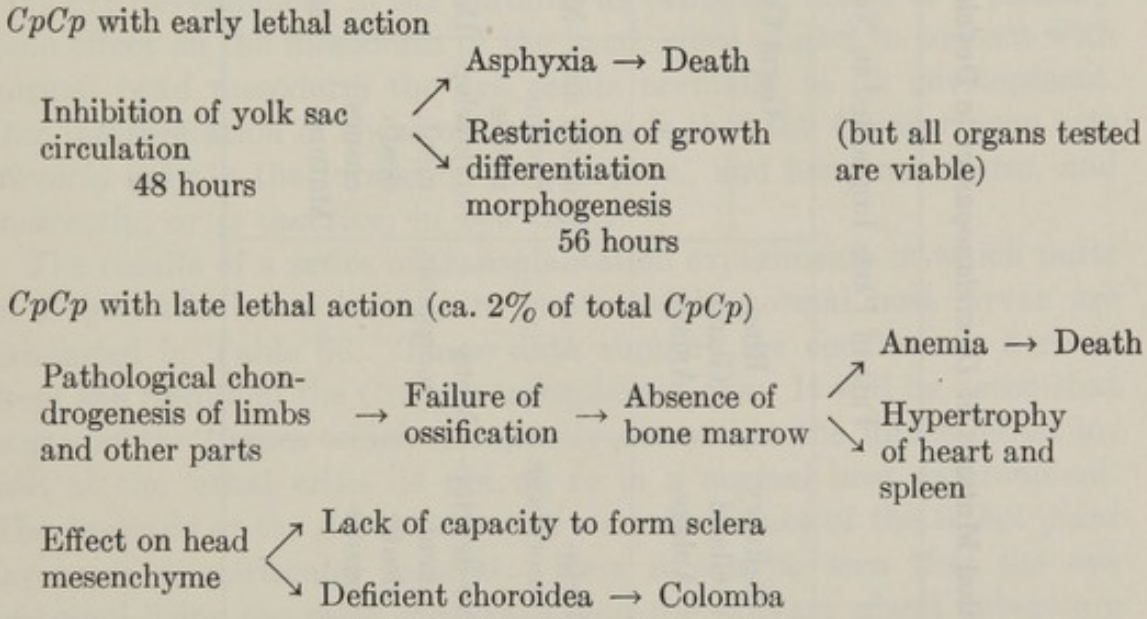


Fig. 116. Action of homozygous creeper in the fowl. From Hamburger (254).

normal hosts, David (130) was able to demonstrate that the lethal action of the *Creeper* gene is superficially non-autonomous in its effect. All tissues tested, such as heart, presumptive cartilage, etc., survived and grew for periods long past the time they would have been expected to cease growth had they been left *in situ*. Presumptive creeper limb bud materials from embryos as young as 30 hours old, however, do not develop into normal limb buds but clearly retain the *Creeper* characteristics (254, 523). This is true for limbs derived from both homozygous and heterozygous donors. Although the development of the limb bud is clearly autonomous, that of the eye in the homozygous mutants can clearly be shown to be heteronomous. Homozygous *Creeper* eye primordia transplanted into normal host sites after the removal of the host eyes develop normally despite their genotypic constitution (254).

The *Creeper* gene has a definite pleiotropic effect as shown by Fig. 116, which is accentuated if the embryo succeeds in surmounting the

Table 53. The Ability of Various Parts of Certain Lethal Mutants of *D. melanogaster* to Develop in Normal Hosts *

Mutant	Maximum Developmental Stage of Whole Insect	Imaginal Discs (Eyes)	Fate of Parts Implanted in Normal Host				
			Salivary Gland Anlage	Ring Gland Anlage	Germ Cells		Somatic Tissue of Gonads
					♂	♀	
Translucida *l*(3)*tr*	Partial, but abnormal development of imagos in puparium	Metamorphose	Metamorphose	—	Mature and become motile	More or less mature	Metamorphose
Lethal giant larvae *l*(2)*gl*	No pupal development, only formation of puparium	No development, death ensues	No development, death ensues	Metamorphosis	No development, death ensues	Develops normally then dies	Metamorphose
Meander *l*(2)*me*	Death in 3rd instar	Metamorphose	—	—	Mature	Mature but sterile	Metamorphose

* Data from Hadorn (244).

original difficulty of poor yolk sac circulation. Of the two major manifestations of the mutant gene in the embryos which succeed in developing to the end of incubation, the effect on limb formation appears to be definitely more direct than that on the eyes, because the limbs develop autonomously. Thus it may be assumed that the mutation has primary effects on limb formation presumably by interfering with the normal course of cartilage and bone formation, and secondary effects on the eyes. Hamburger (254) interprets the aberrant eye development in the mutants as being the result of a primary gene effect on the mesoderm of the head, since placed in contact with normal head mesoderm the eye reacts normally in its development. One interpretation of these observations is that the *Creeper* gene acts *directly* only in the formation of bone, etc., and head mesoderm, and *indirectly,* or *is inactive,* in eye tissue.

The results of a series of transplantation experiments in which parts of *Drosophila* lethal larvae are implanted in normal host larvae are tabulated in Table 53. These data support the conclusions derived from the results of the *Creeper* transplantations. It will be noted that many of the tissues which necessarily die within the intact lethal insect at the lethal crisis do not do so in a normal host environment. The example of the development of various tissues of the *lethal giant* larvae is of particular interest. Here it can be seen that the eye imaginal discs, the male germ cells, and the salivary gland anlage are quite incapable of developing in a normal environment, but the ring glands and the somatic tissue of the gonads continue to differentiate to the imaginal condition. The female germ cells are partially affected in the normal host, since they develop beyond the point which they would in lethal larvae but die before maturity.

CHAPTER 14

Genetics, Development, Nutrition, and Disease

Change in an organism may come about through mutation, or as a result of fluctuation in environment. Neither of these factors is strictly independent of the other, since both influence patterns of metabolism and each limits the degree to which the other can alter a given pattern. Although mutation is a sudden event, it can produce almost any degree of effect from those barely detectable by known means to those too extreme for a cell to survive. Such differences in degree are the result of the extent of change in the mutating hereditary factor and to the influence of the existing genetic background. The environment too can produce metabolic changes of various magnitudes. But the question arises: in the absence of mutation and in a constant environment, is it to be expected that the metabolic processes of a cell will remain constant? Under such conditions, is it to be expected that relative reaction rates will not change and that there will be no accumulation or depletion of any metabolite?

The discussion in Chapter 13 has shown that organisms and their cells are never static but always changing in time. If cells did not change, there would be no cell division, development, aging, or death without mutational or external environmental fluctuations. It seems most likely that biochemical systems are never quite balanced and that there is usually a "metabolic drift" of a constant or variable rate to give rise to accumulations or depletions of metabolites and thus to various threshold phenomena that are characteristic of life processes. Perhaps it is through such "metabolic drifts" or differentiations at the molecular level that cells grow and divide to give new cells that in turn repeat the same processes. This is a subtle aspect of the problem of the relations of genetic constitution to nutritional

requirements and to susceptibility to disease. It is nevertheless a very important one which introduces a time factor into the picture and provides a basis for a differential response to imposed conditions without mutational or environmental changes. It must be assumed, of course, that the potential directions and magnitudes of "metabolic drifts" are limited and controlled by genetic and environmental potentialities.

Various ways by which gene and environmental changes influence metabolic patterns have been the subject of extensive discussions of earlier chapters in this book, and it is to be expected that the same principles are applicable if and when the phenomenon of metabolic drift occurs. As emphasized previously these factors are understandable only through information on what metabolic reactions take place and the relative rates by which they proceed *in vivo*. In the absence of such extensive information it is only possible to suggest qualitative relations between genetics, development, nutrition, and disease, and to see how some of the contributions that have been made fit into this general approach to the problem. At the outset it is well to note that generalizations must be approached with great caution, particularly because of the fact that genetic homogeneity is extremely difficult to attain and maintain in any experimental material.

Frequently individual differences that occur may be detectable at the molecular or reaction rate level only, under a given set of conditions. Thus individuals frequently have very similar phenotypes, but for different biochemical reasons, and it is obvious that undesirable characteristics of a like appearance are not necessarily correctable by the same means. In studies dealing with problems of nutrition and disease investigators are prone to disregard exceptional cases and to accept averages and "normal" ranges as solutions to their problems. This approach has been, and is still, a practical necessity, but the facts of existence of ranges and exceptional cases points to the need for examining the characteristics of individuals. As discussed to some extent later, an organized and extensive program of this sort has been undertaken by Williams and collaborators (690, 691). There can be little doubt but that studies of this kind will eventually provide more exact bases for diagnosis and treatments of unfavorable conditions in individuals.

1. Inherited Anomalies

Mutations frequently result in inherited changes that are sufficiently far from "normal" as to attract the attention of investigators and to arouse interest in the possibility of finding means for correcting deleterious effects. Such inherited anomalies have been studied very extensively in microorganisms, as, for example, the nutritional mutants discussed particularly in Chapters 5 and 8. These mutational effects would be lethal in inadequate environments, but in the presence of appropriate metabolites survival is possible and growth within the normal range is often obtained. Still, as emphasized previously, a mutation and concurrent loss of biochemical capacities remains a characteristic of a strain even though "normal" growth can be obtained in the presence of a single added nutrient. It might be possible to restore completely a premutational pattern of metabolism even including that concerned with gene reproduction and gene function by addition of the right components to the environment, but it is quite clear that a great many cells do not have mechanisms for transporting all the necessary substances through membranes to the appropriate position within the organized system. It might reasonably be presumed that the simplest way to restore desired characteristics to a mutant cell would be to add back a normal gene from the environment. This would be a most satisfactory nutrient, but means for doing it are not ordinarily available. Still, it is not beyond possibility, as indicated by the extensive investigations on transformation and transduction in bacteria (723*a*, 611, 14) and other transfers of heritable characteristics by means of subcellular entities (573, 376). These, in effect, amount to what we usually call infections, and as such can be deleterious, but there is no reason why they cannot be made advantageous as they are known to be in some animal forms. It remains to be seen whether such an approach is feasible in higher organisms with a high degree of differentiation. They are certainly susceptible to infection by some kinds of subcellular units such as viruses.

The problem of correction of known inherited anomalies in higher organisms is not necessarily more complex than that in microorganisms. It often appears so only because mutants of the latter class are frequently spontaneous and only semilethal so that correction is not necessary for survival. As noted previously the mutants of microorganisms that have been studied most were selected on the basis

of correctability of the lethal effect of mutation, and most of the semilethals and strains which do not respond to imposed environments have been discarded. This kind of selection has not been used appreciably in the higher organisms, and means for alleviation of unfavorable effects of mutation are not known in most cases, although, of course, many lethals can be carried in the heterozygous state in diploid organisms or even as heterocaryons in some haploid organisms (p. 17). Actually it is possible to correct, at least partially, the deleterious effects of unfavorable genes or groups of genes in higher organisms, even in man. Some examples of these are discussed in subsequent pages. One important characteristic of many of the deleterious mutations in higher organisms arises from the fact that their obvious effects are intimately bound up with processes of development. That is, an individual may appear to be normal up to some particular stage of development and then suddenly die or develop abnormalities.

Some Lethal Mutants of *Drosophila*

Hadorn and collaborators (245, 246) as well as others have investigated many of the *Drosophila* lethals in relation to developmental patterns. As shown in Table 52 many different stages of development are affected characteristically by specific mutations. At least the lethal effects occur at different stages, although it seems highly probable that this is a matter of degree of effect and that biochemical investigations will show the presence of abnormalities at all stages.

Examples of mutations which produce death very early after fertilization or at various times after hatching from the egg up to the adult stage have already been discussed in Chapter 13. In all cases there is some variation in the degree of effect of the mutation. Some individuals are less normal than others prior to the time of death, and others may seriously be affected at the proper time but still survive the critical period to succumb at a later sensitive period. Some may even develop to the adult stage. Hadorn has discussed this "breakthrough" phenomenon in connection with various influences on penetrance of the genes involved with the suggestion that the lethals are all "phase-specific." It is as though development presents a series of barriers which must be overcome if the process is to continue.

This is a reasonable and useful concept which leads to the suggestion that the critical phases consist of rapid shifts in rates or emphasis of portions of metabolic patterns, and that before, and perhaps after,

a critical stage the portion most affected by mutation is not sufficiently important to the whole to cause a serious deviation from normality. This is in keeping with the concept that the abnormality is present at all times, even though, as in nutritional mutants of microorganisms, the condition can be corrected sufficiently for survival and approximately normal appearance. It is not a simple matter to investigate, but it is apparent that much can be done using the material in *Drosophila*. As an example, Gloor (201) observed that the *l*(3)*tr* larvae contain a very low percentage of globulin in the hemolymph. It was subsequently observed that this mutant accumulates large quantities of free amino acids and peptides in the body fluids (201). This suggests that the mutation results in an anomaly in protein synthesis at the critical stage in development.

Some Inherited Anomalies in the Mouse

A great variety of inherited abnormalities are known in the mouse, and many of them have been analyzed extensively on an embryological basis (204, 239). This approach has yielded much information concerning the varieties of effects of a mutation on different tissues. A few examples of anomalies in the mouse are summarized briefly in Table 54. Two of these inherited characteristics, *rhino* and *pituitary dwarfism,* can be altered in the direction of normality by external means: the first nutritionally with vitamin A, and the second by transplants of normal pituitary tissue. A complete correction of the disorder is not achieved in either case. The yellow lethal provides an example of a mutation that has a lethal effect very early in development while the hydrocephalus characters demonstrate similar phenotypes due to different genes and probably quite different biochemical origins. Gluecksohn-Waelsch (204) has emphasized that similar phenotypes arising from different mutations in mice are common and each case must be examined on its own merits and in relation to developmental processes. This approach has been used extensively by several investigators. Grüneberg's (239) analysis of the hydrocephalus character due to the *ch* mutation (Table 54) is summarized in Fig. 117. Here it is considered that the primary effect of the mutation to *ch* is on the process of cartilage formation during early development and that the subsequent errors in various tissues and structures are all a reflection of this primary disorder. A further deduction has been made that seems especially important to this discussion, but which cannot, at present, be supported by experimental facts. This

Table 54. Some Inherited Anomalies in the Mouse

Character	Gene	Expression
Yellow lethal	A^y	Failure of embryo implantation in the uterus. Markedly affected by genetic constitution of both embryo and mother.
Flexed	*fl*	Causes transitory anemia in newborn beginning 13 days after fertilization. Apparent failure of liver hematopoeitic function corrected after bone marrow starts hematopoeitic function. Also gives fused tail vertebrae and retardation of early embryonic growth.
Rhino	hr^{rh}	Hair abnormality with follicular hyperkeratosis associated with dipilation. In grafts, hair adjacent to normal is normal. Partial correction with large doses of vitamin A.
Pituitary dwarfism		Growth ceases after 2 weeks. Slow differentiation. Sterility. Anterior lobe of pituitary abnormal. Growth and sterility corrected by pituitary implants but pituitary remains abnormal.
Danforth's short tail	*Sd*	Heterozygote has small or no kidneys and homozygote no kidneys and lethal. Short or no tail and other vertebrae fused, abnormal, or missing. Tail normal to 10 days and then tissue degenerates.
Hydrocephalus	*hy* and *ch*	Abnormal development of bones of the head and other effects. *hy* appears to arise from abnormalities in early embryonic fluid circulation and *ch* from a cartilage anomaly (see Fig. 117).

is that the primary anomaly in *ch* is in the cells which give rise to cartilage. It would be expected that, on a biochemical basis, other kinds of cells would also be affected since all presumably have the same genotype. In spite of this some cells and tissues appear to be normal. This difficulty can be rationalized by an extension of the suggestion made previously in connection with the *Drosophila* mutants (p. 293). It is clear that different cells of the same organism have different patterns of metabolism (and thus different potentialities for metabolic drift), and it seems probable that the specific portions of the patterns most affected by a mutation such as *ch* are more prominent in the economy of the types of cells which give rise to the observed phenotypic abnormalities. On this basis the mutation has an effect on all kinds of cells, at all times, to some degree. Major abnormalities may then appear at critical stages of rapidly shifting metabolic patterns such as must occur during differentiation or as an ultimate result of metabolic drift in an aging system.

Related, perhaps, to these critical stages are the "sensitive periods" discussed in Chapter 11, in connection with the effects of temperature on the phenotype. These are periods in which the phenotype can be altered noticeably by affecting the course of development with a particular type of stimulus, which when applied at other periods may have no effect, or a different one.

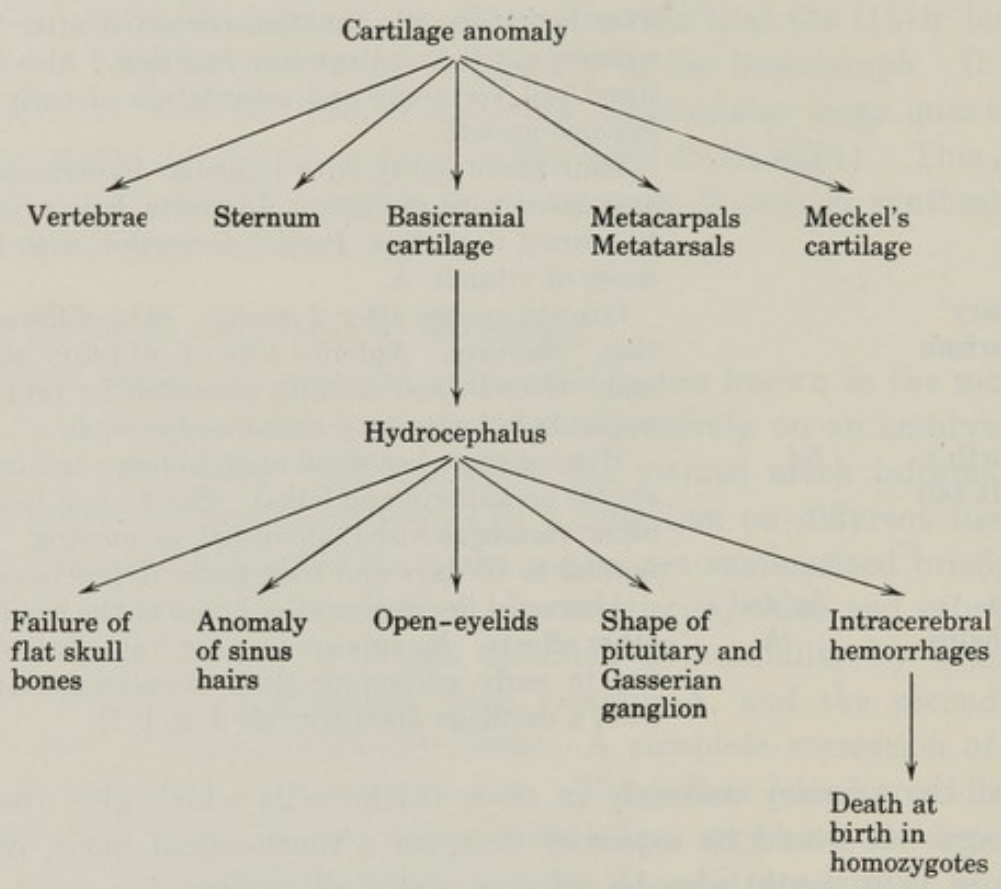


Fig. 117. A pedigree of causes of the hydrocephalus condition in mice.

As emphasized by Little (382) it is in the mouse that the greatest amount of information is available on the genetic aspects of cancer. Some of the known facts concerning this problem are summarized below. The incidence of tumors in mice, whether they be transplants, spontaneous in origin, or induced, is a function of the genetic constitution of the host. The inheritance of the susceptibility to transplants can be reduced to a few or even one genetic factor by inbreeding and selection but it is also dependent on the constitution of the tumor tissue itself. In general, the occurrence of spontaneous and induced tumors is dependent on the presence of an unknown number of genes in the appropriate combinations and the characteristics of the tumors vary with the particular combinations that exist. Thus, the inbred

line of mice C3H gives rise to 97% mammary tumors in virgin females and 90% in breeders while the *A* (Bittner) strain gives 4% in virgins and 78% in breeders (382). Strains which give rise to lung or adrenal tumors and to leukemia are also known. It is clear that there are hormonal effects as in the two strains of mice cited above, and it is also clear that there are maternal effects such as in the cases where a mammary cancer-inducing agent is transmitted to the young through the mother's milk. In a sense these are further expressions of the multigenic and multiple effect character of inheritance of tumor susceptibility.

With regard to the question of the origin of cancer in the presence of a favorable genotype little can be said that is conclusive. Cancer arises through a change in cell characteristics that are transmitted through many cell generations and on this basis it may be considered as due to mutation. Indeed, a number of mutagenic agents are also carcinogenic (X-rays, ultraviolet, nitrogen mustards, dibenzanthracene, etc.), a fact that suggests a somatic mutational origin for cancerous tissue. This is not, however, an established experimental finding.

These general observations regarding multigenic inheritance of susceptibility to cancer may appear to be in sharp contrast to the apparent single factorial inheritance of a variety of characteristics of the mouse as discussed previously, but actually the contrast is not as great as it seems. The expression of the "single factor" mutations is frequently modified greatly in varied genic backgrounds (see Chapter 10), and numerous heritable characters are known in which no one mutant gene produces a sufficient phenotypic effect under all conditions to be recognized. In one genetic background it may appear as a single factor effect, and in another, one component of a multigenic character. With reference to a pathological state such as in cancer it is necessary to recognize the complexities introduced by non-uniformities in genetic constitution. Cancer in a heterogeneous population is a disease of the individual, and even in inbred populations similar tumors of independent origins need not be identical and thus not responsive to the same treatment, at least to the same degree.

Inherited Anomalies in Humans

We are, quite naturally, especially interested in the nature of and means of alleviation of inherited disorders in the human race. A great number of such disorders are known (192, 259, 607), and some of them such as alcaptonuria, sickle-cell anemia, idiopathic methemo-

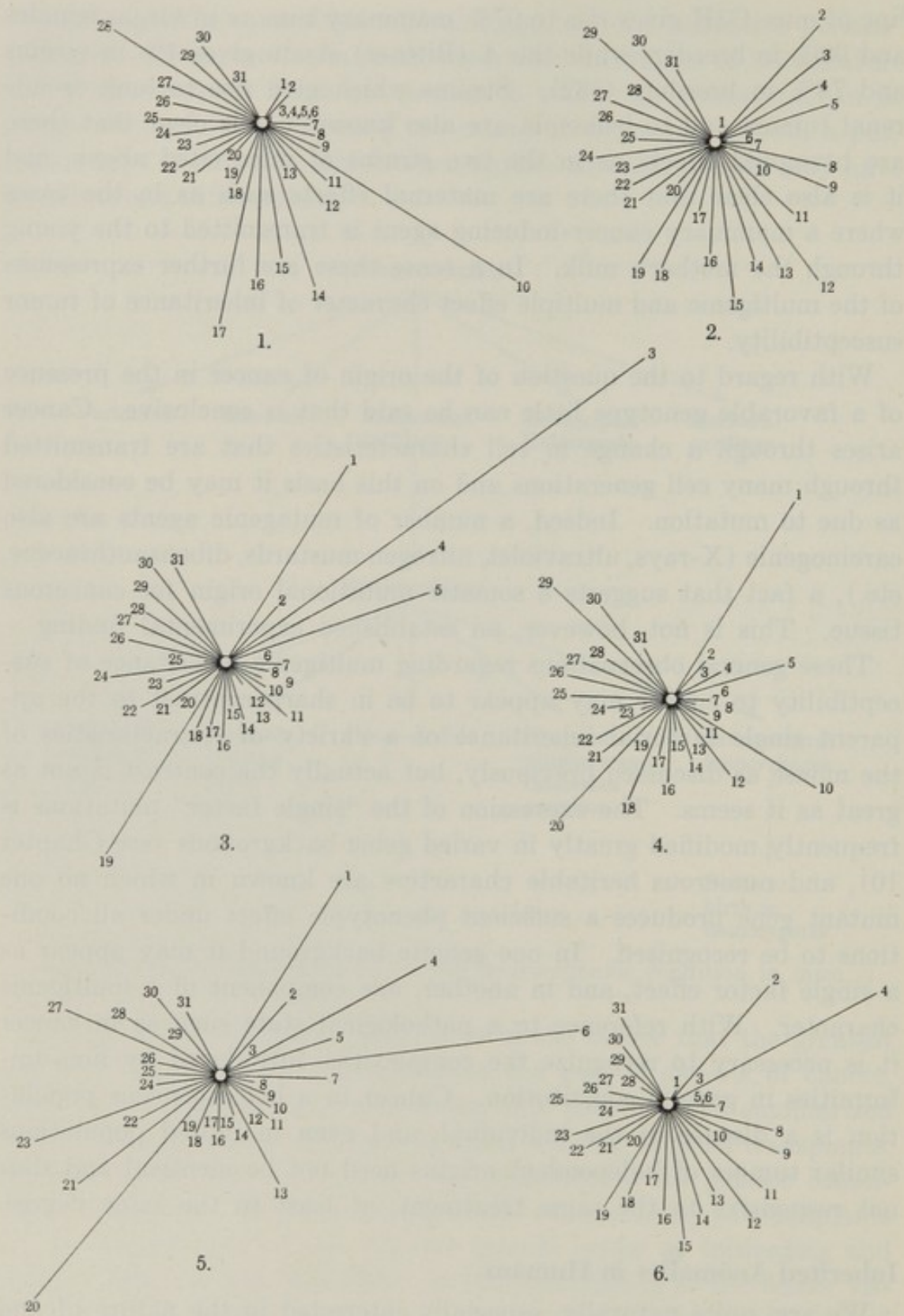


Fig. 118. Individual patterns for humans showing characteristics of taste sensitivities and concentrations of salivary and urinary constituents. The lengths of the polar coordinates indicate the relative amount of the various constituents for each individual. Notice the similarity of the patterns for individual numbers 11 and 12, who are identical twins.

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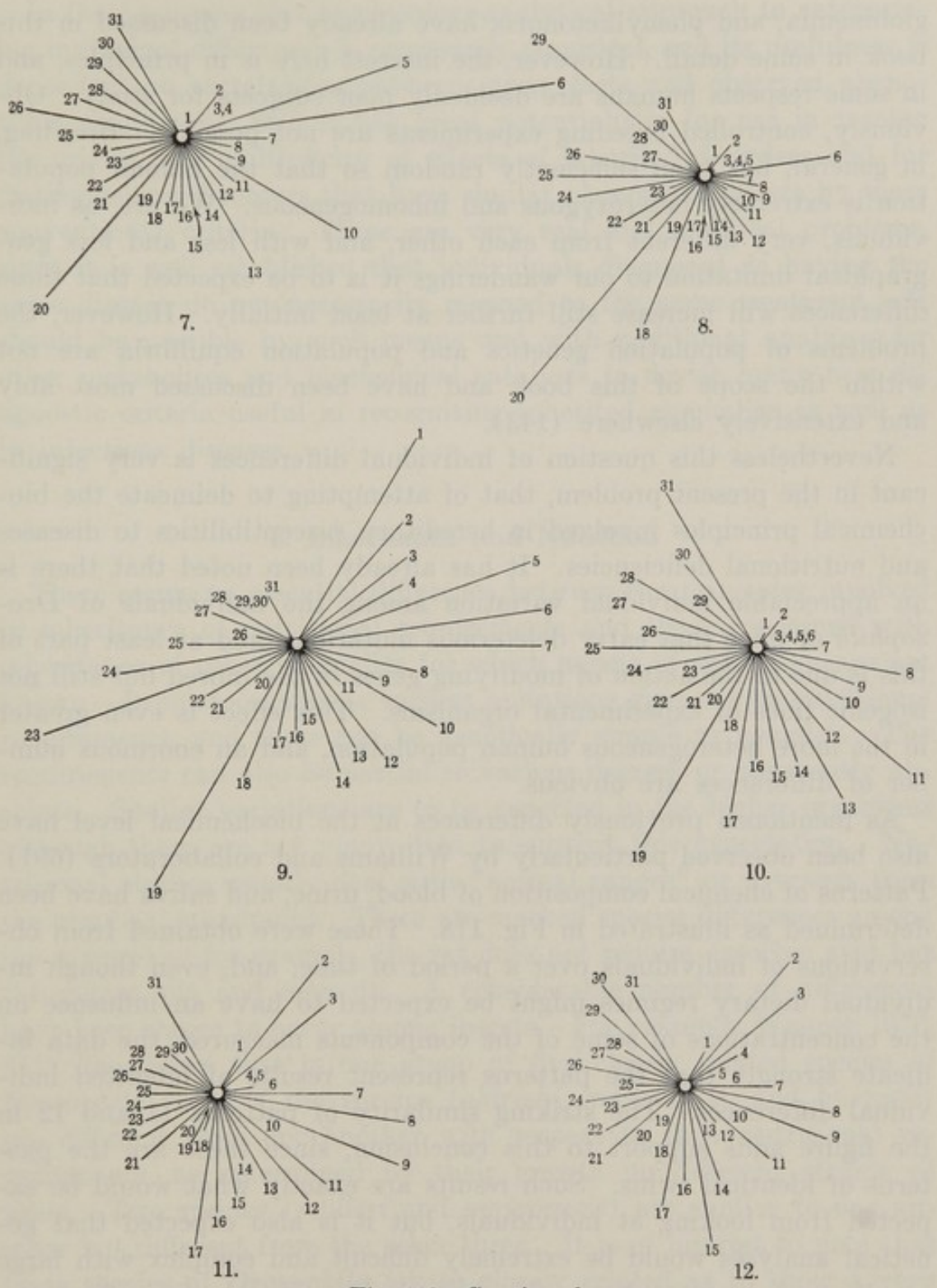

Fig. 118—*Continued*

Taste sensitivity: 1, creatinine. 2, sucrose. 3, KCl. 4, NaCl. 5, HCl. Salivary constituents: 6, uric acid. 7, glucose. 8, leucine. 9, valine. 10, citrulline. 11, alanine. 12, lysine. 13, taurine. 14, glycine. 15, serine. 16, glutamic acid. 17, aspartic acid. Urinary constituents: 18, citrate. 19, base Rf 0.28. 20, acid Rf 0.32. 21, gonadotropin. 22, *p*H. 23, pigment/creatinine. 24, chloride/creatinine. 25, hippuric acid/creatinine. 26, creatinine. 27, taurine. 28, glycine. 29, serine. 30, citrulline. 31, alanine. From Williams (691).

globinemia, and phenylketonuria have already been discussed in this book in some detail. However, the interest here is in principles, and in some respects humans are decidedly poor subjects for study. Obviously, controlled breeding experiments are not possible. Breeding, in general, has been sufficiently random so that the human population is extremely heterozygous and inhomogeneous. We are, as individuals, very different from each other, and with less and less geographical limitation to our wanderings it is to be expected that these differences will increase still further at least initially. However, the problems of population genetics and population equilibria are not within the scope of this book and have been discussed most ably and extensively elsewhere (143).

Nevertheless this question of individual differences is very significant in the present problem, that of attempting to delineate the biochemical principles involved in hereditary susceptibilities to diseases and nutritional deficiencies. It has already been noted that there is an appreciable individual variation among the individuals of *Drosophila* or mice that carry deleterious mutations, and at least part of this is due to the action of modifying genes in the inbred but still not isogenic lines of experimental organisms. This effect is even greater in the more heterogeneous human population, and an enormous number of differences are obvious.

As mentioned previously differences at the biochemical level have also been observed particularly by Williams and collaborators (691). Patterns of chemical composition of blood, urine, and saliva have been determined as illustrated in Fig. 118. These were obtained from observations of individuals over a period of time, and, even though individual dietary regimes might be expected to have an influence on the concentrations of some of the components measured, the data indicate strongly that the patterns represent results of inherited individual differences. The striking similarity of patterns 11 and 12 in the figure adds support to this conclusion, since these are the patterns of identical twins. Such results are exactly what would be expected from looking at individuals, but it is also expected that genetical analyses would be extremely difficult and complex with large numbers of genes and specific gene combinations having effects on particular composition differences. Somewhat similar analyses have been carried out with mutants of *Drosophila* (247) with a demonstration of major composition alterations due to single mutations, but individuals have not been examined for quantitative differences. It would be interesting to do this with such material.

In the beginning this biochemical-analytical approach to categorizing individual differences is necessarily empirical, and its usefulness is dependent on correlations between many facts and observed phenotypic conditions. Still it has great potentialities for use in tracing the origins and multiplicity of effects of inherited disorders and for distinguishing disorders that have similar phenotypic effects by more conventional criteria. These are very real and practical problems, since it is well established that individuals diagnosed as having the same disease do not necessarily respond to the same treatment. It should be possible by such means and with additional analyses for other metabolites and biochemical catalysts to devise many new diagnostic criteria useful in recognizing inherited anomalies as well as in infectious diseases.

2. Inheritance and Nutrition

There seems no essential difference between the principles involved in inheritance of nutritional requirements and those concerned with inheritance of other anomalies for which means of correction are not known. In microorganisms various mutations give rise to nutritional requirements, and these can be genetically simple or complex. The requirements can also be partial to various degrees or apparently absolute. Similar variations are to be expected in the higher organisms although there are not many data to support this expectation. Nutritional studies usually deal with "normal ranges" or averages from the practical standpoint. There are marked species differences among the mammals; for example, the rat does not require ascorbic acid but the guinea pig and man do. A considerable number of differences have been shown to occur among insects. For example, Wagner (670, 671) has shown that in one group of five closely related species of *Drosophila* three of the species (*aldrichi, mojavensis,* and *buzzatii*) are different from one another with respect to their nutritional requirements, as determined by their growth on different strains of yeast. Two species (*mulleri* and *mojavensis*) are similar to one another but different from the other three. It is of interest to note that these species of *Drosophila* are so closely related as to show a very high degree of morphological similarity, and a considerable degree of cross fertility in interspecific crosses.

Practically no reports exist of mutation in mammals to produce a simple absolute nutritional requirement which does not already exist. Perhaps the specialization required for developmental processes pre-

clude this as a possibility, but on the other hand the necessary experiments may not have been done. It would be a considerable task. Elson (158) and Koller (332) have reported briefly on the relative effects of thiamine deficiency in several inbred lines of rats. Using the strains MRC, Strong A, C_{57}, CBA, and C3H it was found that there are marked differences between strains on a thiamine-deficient diet with respect to survival time, activity of the succinoxidase systems in the livers and in cytological abnormalities in spermatogenesis. Such results point to inherited quantitative differences in requirements for thiamine, and it is probable that many levels of requirement would be found in a heterogeneous population of rats. As mentioned previously vitamin A partially alleviates the deleterious effects of the *Rhino* mutation in mice, and this appears to provide a clear case of mutation to a nutritional requirement in mammals. With respect to the insects Hinton et al. (279) have described a mutant of *Drosophila* which needs adenine as a dietary supplement under certain conditions. It is also to be recalled that the mutants of *Drosophila* that can make use of kynurenine and hydroxykynurenine to restore a normal eye color can be classed as nutritional mutants, as can mice with pituitary dwarfism which respond to hormones, and diabetic humans who respond to insulin.

These relatively limited observations on inheritance of nutritional requirements in higher organisms are quite in line with expectations based on studies with microorganisms. In all cases alleviation of inherited disorders by nutritional means does not eliminate the bad genes or combinations of genes. It merely counteracts their effects. One approach to counteracting the effects of poor inheritance, in general, is to supply excessive dietary amounts of known important metabolites. This has been used successfully by Williams (690) and others in the treatment of alcoholism in man by means of high-B-vitamin supplements. It appears that the tendency to become alcoholic is inherited, but it is probably multigenic and it may arise from a variety of different gene combinations. In fact, every individual alcoholic is probably appreciably different, and it would be surprising indeed if all of them could be aided by one treatment such as by the vitamin supplement. Still, this approach can be useful and perhaps applicable in other types of drug addictions.

There are definite limitations, however, to what can be done with dietary supplements since it is well known that some and perhaps most metabolites become inhibitory in high concentrations. In the lower organisms it has been shown that some mutations greatly in-

crease the sensitivity of the mutant strains to inhibition by normal metabolites (p. 223), a fact which emphasizes the need for knowledge of the proper balance of dietary nutrients especially when they are given at a high level.

3. Symbiosis and Incompatibility

The discussion up to this point has dealt largely with the interaction of individuals with their non-living immediate environments, although such isolation is rarely, if ever, attained. The various classes of organisms are frequently very much dependent on each other for nutrient materials, and symbiosis either at a distance or in very close association is a phenomenon essential to continued existence of innumerable species. Animals, for example, depend on plants for the production of carbohydrates and on plants and microorganisms for the production of the nutritionally essential aromatic amino acids such as phenylalanine and tryptophan. Here the association between the organisms involved need not be very close, but in a great many cases physical nearness is necessary. For example, the ruminants depend on the action of microorganisms of the digestive tract to make available the carbohydrates of the plant cellulose ingested, and termites require the assistance of symbiotic Protozoa in order to make use of a good part of their woody diets.

In such cases as these, and there are very large numbers of them, the organisms involved are not only compatible but even interdependent, sometimes almost to the same extent as the interdependence of different tissues of the same organism. From this extreme, organisms exist together at various stages of decreasing compatibility until at the other extreme they become so antagonistic as to remain completely aloof or to militate against survival of one or both when in close association. Frequently dependence and incompatibility exist simultaneously often to the detriment of either or both organisms involved. Infectious diseases in general fall in this category. These have dependence, antagonistic and resistance qualities with characteristics depending on the genetic constitutions of the associated organisms. The existing environment and the potential for "metabolic drift" are important factors in determining the nature of the interaction and the course of the mutual association over a period of time.

Such situations encompass a great number of present and potential variables. Those which include adaptation and mutation provide a basis for constant selection of individuals which can give rise to pop-

ulations of organisms with new characteristics. It is immediately obvious that the problems associated with an understanding of the interrelations between organisms are similar to, but more complex than, those concerned with the biochemical nature of inheritance in one organism. Nevertheless a great deal of work has been done in this field, and some examples and discussion are presented in subsequent pages.

Bacterial Virus

A most interesting and instructive example of the influence of heritable factors on infection is found in the recent and current work on phage-bacteria interactions. Phages or viruses that infect bacteria are very common, and there are very large numbers of different kinds, found, for example, in sewage water. Only a few of these have been studied intensively. Many special methods have been devised for studies of inheritance in phages as well as in the host bacteria. Even though the mechanisms of passage of units of inheritance from one generation to the next seem to be quite different from those of the higher organisms, it is clear that important principles have been ascertained by studies of such systems (273, 274, 384).

The bacteriophage is a complete parasite on the host since it multiplies only within the bacterial cell. It is a small (about 20 to 100 mμ in diameter), organized body which, in general, has a protein-lipid membrane containing a quantity of DNA and perhaps a small amount of other materials. Many of the phages have a more or less spherical head and a long slender tail; others are tailless. Different strains can be distinguished quite satisfactorily by morphology, antigenicity, and reactions with host bacteria which have different genetic constitutions. Some of the general characteristics of the phage-bacteria relations may be summarized as follows. One or more phage particles become attached to the bacterium, those with tails becoming attached by these structures. The contents of the phage membrane are then transferred to the bacterium, and the membrane or ghost remains outside. This can be shaken loose, and it does not participate further in the production of new phage particles. However, the ghosts, a number of whole phage particles, or even the unrelated synthetic polymer of dimethylaminoethyl acrylate can induce lysis of a bacterial cell from without under certain conditions, apparently through their influence on the activity of bacterial surface enzymes.

This process does not yield phage. Single phage particles can produce infections but they do not induce lysis from without.

When infection occurs, and immediate lysis does not, the bacterium is still "killed" so far as its own reproduction is concerned if the bacterium is genetically sensitive. From this point it becomes devoted to the production of new phage particles in collaboration with the material that was transferred from the infecting phage into the bacterial cell. The particles which become phage (vegetative phage) are produced in numbers from about 30 to several hundred per bacterium, and during this process something equivalent to mating takes place. If phage particles having different heritable characters are present, recombination occurs with results simulating randomness in a small population. The different characters can arise through a double infection or mutation of the vegetative phage. Following reproduction and recombinations new phage membranes are formed and the bacterial cell bursts to release mature and infectious phage particles which can repeat the cycle with other bacteria.

Infection can take place in resistant bacteria, but multiplication of the vegetative phage does not occur although it can be carried along indefinitely by the bacteria. Phage in this condition are termed lysogenic. Their multiplication and host lysis can be induced by radiation or chemical treatments. It also happens spontaneously with a low frequency. Lysogenic phage is detectable only by this appearance of lysis and new phage particles. The infected resistant bacteria multiply and carry on their metabolic processes in an apparently normal fashion. This is in striking contrast to the changes which accompany the infection of sensitives. In this case as noted the bacteria do not reproduce and they appear to devote all their metabolic energy to phage reproduction. Their own substances, at least the DNA, disappear and become converted to phage DNA.

The various steps that occur during the process of a phage infection of *E. coli* have been clearly defined (498). The initial attachment is due to a reversible formation of electrostatic bonds between specific sites on the virus and host. The properties of these sites are inherited characteristics. The phage membrane and its contents are then separated and the process becomes irreversible. The nature of the infective process itself varies with the characteristics of the bacterium and virus. Interactions of heritable systems give rise to lysogenicity or to vegetative phage multiplication. Little is known about the details of these processes or those concerned with maturation of the phage

particles. It is clear, however, that many stages and processes are influenced by genetic constitution.

In some respects these experimental findings concerning the interaction between phage and bacteria provide more precise details on the nature of infections in relation to genetic constitution than do systems involving higher organisms. Here the phage itself (but perhaps not the vegetative phage) appears to be quite inert, and without a capacity for carrying on metabolic processes; yet it contains determiners of heredity seemingly in a linear order as in the more autonomous, organized systems of cellular organisms. In more complex systems it is necessary to consider simultaneously more or less independent metabolic patterns of a host and the infecting organism.

There is one further development in connection with phage-bacteria relations that should be mentioned though an extensive discussion is not possible here. This concerns the process of transduction in *Salmonella,* and other bacteria by which hereditary host characters can be transferred from one cell to another (611, 723*a*). It has been suggested on the basis of existing experimental evidence that phage which is produced in a donor cell can incorporate some of the heredity-determining material within its membrane and transfer it to a recipient cell where it replaces the existing homologous material. This process is rare, and the phage involved relatively inactive so far as lysing or killing is concerned. It seems possible that transduction is a special case related to the system already discussed. In that system all the hereditary substance of the bacterium is apparently converted to that of phage. Perhaps in transduction this conversion is not complete. Much less is known about the transduction process than about the coliphage system already described, but further investigations should yield additional valuable information concerning the action of infectious agents and the interactions of the hereditary characters of host and virus.

4. Genetic Factors and Infections in Higher Organisms

There are in the records of plant and animal breeders enormous numbers of examples of practical investigations on inheritance of resistance to diseases (220, 676). Striking success in obtaining resistant strains has been achieved in many cases, as for example in the development of strains of oats and wheat that have resistance to rusts and smuts. Such developments with these and other crops have been of very great economic importance and will continue to be so. This

whole field of endeavor, however, is plagued with the problem of mutation. Host organisms may mutate, sometimes by a single change from resistance to sensitivity and the reverse. The same is true of virulence in the pathogenic organisms that cause diseases. However, resistance and virulence are relative conditions with their expressions depending on environment and usually on the action of a number of genes in each, the host and the infecting organisms. For this reason there is a continuous change and the necessity for constant selection of new host strains in order to keep ahead of unfavorable changes in the pathogens. Under appropriate conditions where a pathogen can multiply rapidly to give large numbers of progeny (that is, in a concentration of sensitive hosts) a single mutation in one individual can give rise to an epidemic. This "battle of mutants" proceeds constantly, sometimes giving rise to very large fluctuations in populations.

Broadly speaking, little is known of the genetically controlled physical and biochemical factors that are directly involved in resistance and virulence, but it is clear that the potentialities are multitudinous. As demonstrated so clearly by the investigations with phage, a host and infective agent must be of the right constitutions for maintaining contact prior to invasion, in order for the process of invasion and subsequent support of the population after invasion to take place. Each of these processes is complex, dependent on the particular patterns of metabolism that are possible and the specialized mechanisms that have been developed for offense and defense.

In animals a good deal is known about defensive mechanisms that aid in resistance to infectious diseases. These include the genetically controlled properties of a variety of systems such as those concerned in the production of antibodies in response to the invasion by foreign proteins, the phagocytes which engulf and digest invaders, and the biochemical systems that produce inhibitors against invading organisms. What is probably a summation of these effects in the resistance of mice and chickens to *Salmonella* (typhoid) as influenced by selection of genetically resistant hosts has been cited by Gowen (220) (Fig. 119). These experiments were carried out by intraperitoneal injections of constant numbers of bacteria followed by inbreeding of the best survivors. Survival under the conditions maintained was less than 20% in the first generation, but showed a rapid rise at the beginning (double or more in the second generation), followed by a gradual leveling off to about 80–90%, as shown in the figure. The dosage of bacteria was then increased, giving some further resistance

selection. Complete resistance was not reached, and apparently this does not occur. These procedures no doubt involve selection for proper combinations of genes that already are present in the parental lines, as well as selection for mutations that occur in the hosts during the course of the experiments. Simultaneously, in each infection there were no doubt selections for mutants of the bacteria, and this may

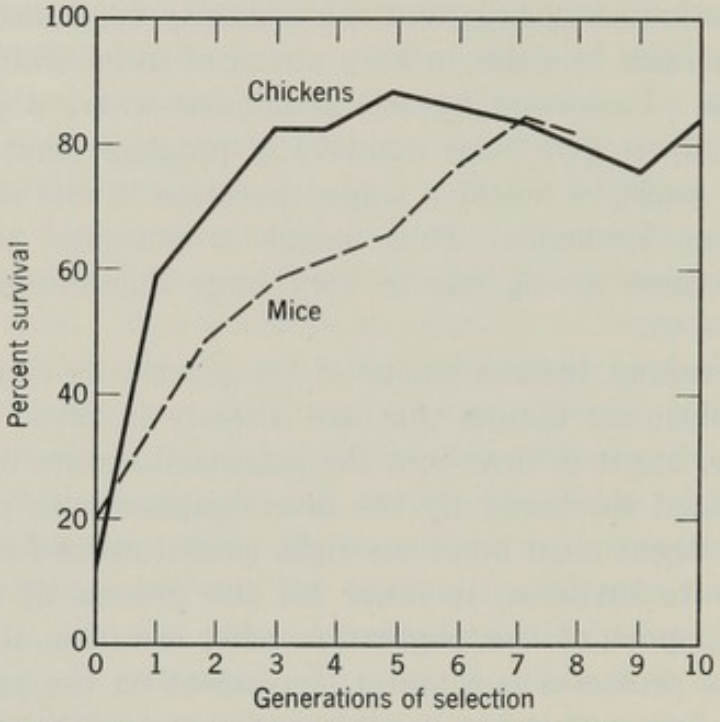

Fig. 119. The development of resistance to *Salmonella* sp. by the fowl and mouse as a result of selection over a number of generations. In neither case does the survival increase over that indicated in the graph; i.e., 100% resistance is not attained even after many generations of selection. After Gowen (220).

account for variations that occur after the resistance leveled off as well as the absence of complete resistance. These results are typical of many obtained from similar experiments, and there is little question but that the interactions obtained are dependent on the combined actions of many hereditary units in both hosts and bacteria.

From other experiments (220) involving inbred lines of mice with different sensitivities to *Salmonella* and lines of the bacterium with different degrees of virulence, further interesting and useful facts were ascertained. A study of the effects of acquired immunity by the different lines of mice was also included in the experiment. Immunization was achieved by periodic injections of heat-killed bacteria, and it was confirmed that greater quantities of vaccine yielded greater resistance. It was also found, however, that the dead bacteria had a

killing action on the most extreme sensitives among the lines of mice. This is reminiscent of the "killing from without" observed as the result of treatment of bacteria with phage, phage ghosts, or a certain synthetic polymer (p. 404). Perhaps similar inherited characteristics are involved. The experiments with mice also show that the bacteria with low virulence are relatively ineffective as immunizers and that the most sensitive lines of non-immunized mice are also the most sensitive after vaccination. The level of resistance in all lines is simply raised by vaccination (1 to 200 fold) but to a degree dependent on the host genotype as well as on the bacterial genotype.

With respect to the specific details on just how infection of higher organisms takes place, or just which gene-controlling biochemical processes are involved most directly, information is decidedly limited. A great deal of work has been done on the problems concerned with invasion and how cells or large units cross membrane barriers. Many cells contain quantities of a gel-like mucopolysaccharide, hyaluronic acid, which acts as an effective barried toward the entrance of foreign materials. This polymer, which does not necessarily have the same composition or structure in different cells, is made up largely of acetylglucosamine and glucuronic acid. Chemical linkages are mainly glycosidic and molecular weights probably higher than 500,000. Insoluble salts with proteins are formed through the acidic groups. Connective tissues contain a somewhat related substance, chondroitin sulfuric acid, which also contains glucosamine and glucuronic acid. In addition the number 6 position hydroxyls of the glucosamine are esterified with sulfuric acid. This substance may have a function similar to that of hyaluronic acid, and indeed some enzyme preparations (hyaluronidase) hydrolyze both substances. Numerous infectious bacteria and such secretions as snake venom and semen contain quantities of the hyaluronic acid-splitting enzyme hyaluronidase. It has been the contention of many investigators that this enzyme has an important function in the breakdown of the mucopolysaccharide barriers that prevent invasion of tissues by foreign materials. Processes of bacterial and viral infections, fertilization, and even invasiveness of malignant cells have been interpreted on this basis. In bacteria hyaluronidase is bound in an inactive form which is activated during the edema accompanying inflammation, indicating that the enzyme itself does not account for all of the invasion process. It is apparently more complicated than the dissolution of the hyaluronic acid barrier. Nevertheless, this system certainly does have a significant role in infection, and it is easy to see how

genetic changes might affect resistance and invasiveness by modifications related to the substrate or to the enzyme.

5. Some General Considerations

This necessarily limited discussion of the hereditary aspects of problems of nutrition and disease does not raise or answer any really new questions that are not considered to some extent in other sections of this book. Emphasis is placed on the factor of change with time and on the importance of considering these problems in terms of individuals. It is of course fundamental to know the biochemical nature of a system developed under the influence of a given genotype, but this can serve only as a basis for an assessment of the potentialities for change. A steady state in biochemical processes is not a characteristic as it exists and evolves. It is for this reason that repeated references are made throughout this book to the importance of *in vivo* rates of biochemical processes. A complete pattern of metabolism, including information on instantaneous reaction rates, is, however, still not enough. The relative changes in rates and corresponding shifts in patterns constitute equally necessary information. That this is so is emphasized by the observations on the etiology of various inherited diseases, and, in a sense, all diseases are inherited because genetic constitution always has an influence on their course. From the practical standpoint genetic heterogeneity is the great enemy of the complete control of disease.

One purpose in writing this book was to attempt to outline, by means of illustrative examples, the experimental findings and established principles that make the fields of genetics and biochemistry inseparable for their ultimate development. The problems posed in general are by no means new, and we have obtained only partial solutions to most of them. For example, in 1932 Sturtevant made the following statement at the International Congress of Genetics.

> It is clear that in most cases there is a chain of reactions between the direct activity of a gene and the end product that the geneticist deals with as a character. One may surmise that any valid generalizations about these reactions are more likely to concern the initial links than the terminal ones. However, it is the terminal ones that are usually open to experimental attack, since the only index to the effectiveness of a given experimental technique is the condition of the end product.

At the present time after many years we are still unable to distinguish with certainty between an "initial link" and a "terminal link,"

and this is a very fundamental problem. Nevertheless we have progressed. We have much more information concerning the mechanisms of inheritance and much more concerning the nature of biochemical reactions. These collected facts now provide a more firm basis for more precise and detailed definitions of problems to be solved even though we still agree with, but have not established experimentally as a fact, the premise set forth in the above quotation. There is no reason to believe that there is any quick solution to the question of how genes control metabolism. The definitions of gene and metabolism have not been completed. It is to be expected that much will come of the various lines of endeavor that are already in progress, and we have contended that special attention should be given studies of factors that influence reaction rates especially *in vivo*.

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Index

METABOLISM