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Progress in

# MEDICAL GENETICS

VOLUME III

Edited by

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PROGRESS IN

# Medical Genetics

Volume III

Edited by

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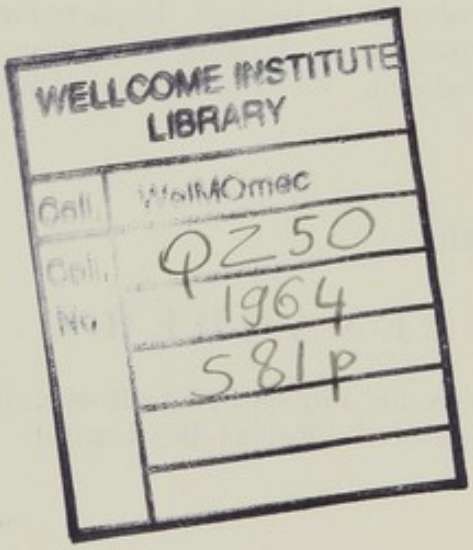


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# Contents

# Foreword

THE DEVELOPMENT OF EXQUISITELY SENSITIVE METHODS FOR THE STUDY OF large molecules, for tracing the path of molecules in chemical reactions, and for the analysis of genetic fine structure in microorganisms has led, with astonishing rapidity, to remarkably detailed information about the nature of the gene, of the genetic code and the role of the code in protein synthesis. This information is currently being applied toward the solution of specific problems in human genetics (Chapter 1).

It has long been recognized that individuals respond differently to a given drug. Similarly, it has long been surmised that this variation is at least in part genetic. The scope of pharmacogenetics, as this subject has been conveniently termed, and its importance for clinicians and geneticists alike is expanding rapidly (Chapter 2). The clinical observation that, on rare occasions, the muscle relaxant suxamethonium caused prolonged muscular paralysis and apnea was the beginning of the fascinating story of the genetics of the pseudocholinesterase variants (Chapter 3).

Surgical techniques have been developed sufficiently so that only immunological differences limit the success of transplants between individuals. Work with experimental organisms is greatly advancing the understanding of the nature of these factors and of the development of methods for their control (Chapter 4).

Advances in human cytology (see Chapter 8, Volume 1 of this series) have contributed greatly to our knowledge of the causes of several syndromes, but they have been most successful in contributing to our understanding of mongolism which is probably the most common genetically determined anomally. The analyses of the remarkable chromosomal patterns and of the physiological effects of this abnormality are among the most exciting recent developments in medical genetics (Chapter 5).

All organisms vary greatly about their "norms" and man may well be the most variable of all organisms. The genetic basis of this variation, except for the extremes, is almost invariably due to the cumulative effects of many genes each of which has so minor an effect that it cannot be distinguished with certainty. This is known as multifactorial inheritance.

The genetics of such characters as blood pressure, and serum cholesterol level in man involves multifactorial inheritance. Methods have been developed in recent years, which permit an insight to be gained into the role of heredity in the determination of such characters (Chapter 6).

The continued testing of nuclear bombs, the expanding use of nuclear energy for commercial purposes and the use of radiation for diagnostic and therapeutic purposes make it important that current information concerning the mutagenic and teratogenic aspects of these radiations and of means for controlling them be available (Chapter 7).

The editors take some pride in their success in persuading this distinguished group of investigators to contribute chapters to volume three of *Progress in Medical Genetics.*

We wrote in the preface to volume one, "It is our plan to maintain the high standards set by these pioneer authors in future volumes of this series." The authors of the chapters in this volume have brilliantly done so.

A. G. S.
A. G. B.

CHAPTER 1

# Chemical Mutagens and the Genetic Code

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"It is not yet known whether all nonsense is absolute nonsense."

F. H. C. Crick, 1963

## I. The Coding Hypothesis

GENETICS IS PERHAPS THE ONLY BIOLOGICAL SCIENCE WHICH LENDS ITSELF TO presentation in a deductive manner. This peculiarity is evident in some of the more elegant descriptions of transmission genetics (Sturtevant and Beadle, 1939) as well as in discussions of the molecular mechanisms of gene action. The following hypothesis, for example, adequately accounts for a large body of experimental information:

1. Genetic information is encoded in the base sequence of deoxyribonucleic acid (DNA).
2. DNA determines the primary structure of proteins. The base sequence of a section of DNA corresponds in a colinear way to the amino acid sequence in the peptide whose structure is determined by that section of the DNA.

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The following abbreviations are used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; A adenylic acid in RNA or deoxyadenylic acid in DNA; G guanylic acid in RNA or deoxyguanylic acid in DNA; C cytidylic acid in RNA or deoxycytidylic acid in DNA; U uridylic acid; T thymidylic acid.

The symbolism A:T or G:C refers to the hydrogen bonded pairing of adenine and thymine or of guanine and cytosine in the two strands of the Watson-Crick DNA structure. A:T is distinguished from T:A. The notation G + C refers to the analytically determined per cent of guanine and cytosine in a sample of DNA.

3. More than one base (pair) must be "read" in order to determine a particular amino acid. The minimum number which now seems adequate is three and the triplet of bases corresponding to a particular amino acid may be called a "codon" (Crick, 1963).

4. The immediate gene product is a ribonucleic acid (RNA) with base sequence complementary to one strand of the DNA. This immediate gene product migrates to the cytoplasm where in association with the ribosomes it serves as a source of specificity for protein synthesis.

5. The rate at which the base sequence is read to make a gene product is under both genetic and environmental control.

Within the past few years astonishing progress has been made in obtaining the evidence on which this hypothesis depends. As Ycas points out (1962), this evidence is far from complete. However, the successes of the hypothesis suggest that there must be points of correspondence between it and reality.

Since it seems evident that groups of nucleotide bases must correspond to amino acids (point 3), one can inquire as to the exact nature of the correspondence. This is the "coding problem." One would like to know: a) what are the base arrangements corresponding to particular amino acids?; and b) how are these individual codons arranged in the gene?; one would like to know what signals the start of a particular gene and what signifies its end.

## II. Evidence for the Hypothesis

### A. *DNA as Genetic Material*

The only satisfying evidence that DNA carries genetic information comes from studies with microorganisms (Avery et al., 1944; Hershey and Chase, 1952). If DNA is prepared from an organism of standard genotype and is then fed under the proper conditions to a mutant organism, transformation of the mutant to the standard type can be observed in a significant fraction of the population. Such transformation has been obtained with *Bacillus subtilis, Diplococcus pneumoniae, Hemophilus influenzae* and with other microorganisms (Ravin, 1961). Numerous characters can be transferred including the ability to make particular enzymes. The process is not considered a directed mutation since several characters can occasionally be transferred together by DNA in a manner reminiscent of the recombination of linked genes in higher organisms. The

transformation phenomenon is therefore thought of as one method of genetic recombination. DNA determines the presence of particular enzyme proteins and presumably does this by carrying information which specifies their structure. Transformation has been demonstrated with human cells using ability to make inosinic acid pyrophosphorylase as a marker (Szybalska and Szybalski, 1962).

Viruses consist of a nucleic acid core surrounded by a protein coat. It is possible to extract the DNA from viruses infecting strains of *Bacillus subtilis*. Such DNA is infectious (Romig, 1962) and induces the recipient cell to produce complete virus (including the protein coat) thereby demonstrating that the DNA contains and transmits the information for particular protein structures. Single stranded DNA obtained from bacteriophage øX174 is infectious but *Escherichia coli* protoplasts must be used to demonstrate infection (Guthrie and Sinsheimer, 1960). Infectious DNA can be obtained from phage λ and this DNA infects intact *E. coli* (Kaiser, 1962). Such experiments demonstrate that DNA can carry structural information for protein synthesis and can assure its own replication. It therefore has the characteristics required of genetic material. It is not to be supposed that DNA is the only substance with such properties (see section III C).

### *B. Genetic Determination of Amino Acid Sequence*

Soon after the development of methods by Sanger which made it possible to determine the sequence of amino acids in protein, it was shown that the insulins from different species differ by amino acid substitutions at particular positions (Harris, Sanger and Naughton, 1956). The hypothesis that these differences were genetic was substantiated by the finding, first by Ingram (1956) and then by others (Keil, 1962); that hemoglobins differing from the normal by particular amino acid substitutions at assorted position in the molecule (Table 1) could be obtained from persons carrying traits inherited as single recessive Mendelian genes. The genetic control of these substitutions was apparent and it was soon widely accepted that genetic control of protein structure was the result of control of amino acid sequence. It is supposed that the three dimensional structure of proteins is completely determined by the primary amino acid sequence (Crick, 1958) but this assumption will not be discussed.

An advantage of microorganisms for studying gene-protein relationships is that it is possible to relate the genetic location of particular mutations to amino acid changes. Many different mutations of the *E. coli* tryptophan synthetase A protein have been isolated and been shown to differ from the wild type by distinct amino acid changes. These mutants

TABLE 1—*Amino Acid Substitutions in Hemoglobin*

| Chain | Residue No. in Chain | Original Amino Acid | Substituted Amino Acid | Mutant Designation: Hemoglobin |
|---|---|---|---|---|
| α | 16 | Lysine | Aspartic acid | I |
| α | 57 | Glycine | Aspartic acid | Norfolk |
| α | 58 | Histidine | Tyrosine | M-Boston |
| α | 68 | Asparagine | Lysine | G-Philadelphia |
| β | 6 | Glutamic acid | Lysine | C |
| β | 6 | Glutamic acid | Valine | S |
| β | 7 | Glutamic acid | Glycine | G-San José |
| β | 62 | Glutamic acid | Lysine | E |
| β | 63 | Histidine | Arginine | Zürich |
| β | 63 | Histidine | Tyrosine | M-Emory |
| β | 67 | Valine | Glutamic acid | M-Milwaukee |
| β | 121 | Glutamic acid | Glutamine | D-Punjab |
| | 9 | Serine | Threonine | D |
| β | 12 | Threonine | Asparagine | D |
| | 22 | Glutamic acid | Alanine | δ, $H_2$ |
| | 50 | Threonine | Serine | |

Data tabulated by Keil (1962) and Smith (1962).

recombine with different frequencies, some mapping very close together, others relatively far apart (Yanofsky et al., 1961). Determination of the amino acid sites affected by different mutations indicates that there is a correlation between the altered site in the protein and the position of the mutation on the genetic map (Helinski and Yanofsky, 1962). This series of experiments lends direct support to the picture of the gene obtained from numerous fine structure analyses (Benzer, 1961). The structural gene can be pictured as that region of genetic material which carries structural information for the formation of a distinct peptide. Within each gene there are a series of sites or codons which specify the insertion of particular amino acids at particular positions along the chain. The sequence of codons corresponds to, or is colinear with, the sequence of amino acids (Fig. 1).

### *C. Complementarity and the Construction of Protein from Subunits*

The *coding ratio* may be defined as the number of nucleotide bases determining the structure of a peptide divided by the number of amino acids in the peptide. If the code is of the nonoverlapping type and if the ratio is three (see below) the size of the gene can be calculated from

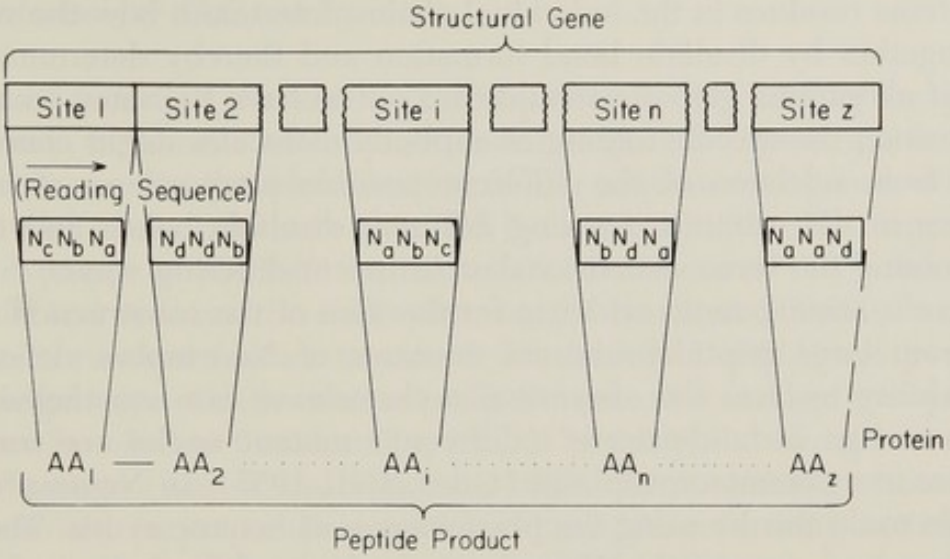


FIG. 1—Structure of the gene. AA = amino acids. Na-d = deoxynucleotide pairs.

the average molecular weight of a peptide chain. The molecular weights of the proteins vary from about 13,000 for ribonuclease through the hundreds of thousands for serum globulins to several millions for some of the viruses when these are considered as single molecules (Fox and Foster, 1957). However, it turns out that many of the proteins are to be considered as built up of subunits joined together by disulfide bonds. Reduction of these disulfide bonds leads to the separation of individual peptide chains of molecular weight on the order of 20,000. Thus tobacco mosaic virus protein consists of subunits of about 17,000 molecular weight and hemoglobin consists of 4 peptide units, two alpha and two beta chains (Rhinesmith et al., 1957), of 17,000 M.W. each. Yeast alcohol dehydrogenase with a molecular weight of 151,000 consists of 4 peptide chains; ferritin with a molecular weight of 500,000 is made of subunits of 16,000 molecular weight (references in Keil, 1962). Mammalian glutamic dehydrogenase is made up of subunits which dissociate under the influence of steroid hormones and the individual subunits have alanine dehydrogenase but no glutamic dehydrogenase activity (Tomkins and Yielding, 1961). Two distinct lactic dehydrogenases are present in the tissues of most animals, one characteristic of heart, the other found in muscle. Both enzymes exist as tetramers. Hybrids of the form M(uscle)$_3$ H(eart)$_1$, $M_2H_2$, $M_1H_3$ can be demonstrated, as though they were formed by random recombination of units within the cell (Cahn et al., 1962).

It is assumed that the genes carry information for the specification of the individual subunits and that proteins are automatically constructed from these subunits without additional genetic information. The location

of cysteine residues in the individual chains determines how the subunits link together by disulfide bond formation and thereby determines folding. (If all gamma globulin should turn out to have the same amino acid composition the specific folding of antibody molecules might conceivably result from selection of the different possible jutapositions of cysteine residues in the subunits, forming different disulfide bonds and thereby determining the three dimensional structure in differing ways.)

There is some genetic evidence for the idea of the construction of protein from large peptide subunits. Mutants of Neurospora deficient in their ability to form the enzyme adenylsuccinase can nonetheless make enzyme when certain pairs of (different) mutant nuclei are combined together in a common cytoplasm (Giles et al., 1957). In Neurospora it is possible to do this by using the phenomenon of heterocaryosis. The mold mycelium is a syncytium. When hyphae of two differing genetic types fuse, their nuclei are pooled in a common cytoplasm. This phenomenon of enzyme formation by pairs of different mutants of the *same gene* is called complementation and has been obtained in a number of organisms and with a number of systems (Woodward, 1960). The nature of the test depends, of course, on the organisms used; in bacteriophage, complementation tests can be performed by simultaneously infecting bacteria with two mutant phage types. In bacteria partial heterozygotes can be obtained by conjugation and transfer of an F factor episome.

The complementation phenomenon has been obtained *in vitro* by grinding together the mycelium of different Neurospora mutants (Woodward, 1959). Such results indicate that the phenomenon is to be interpreted as follows: each mutant nucleus directs the formation of subunits with lesions at different sites. The different subunits recombine in the cytoplasm to give an aggregate with lesions at different places. This aggregate can carry out some of the original functions of the protein at reduced efficiency. All the complementing sites are within the same structural gene and the fact of complementation *in vitro* can be taken as indicating that the construction of protein from subunits can occur without an additional structural gene.

It is therefore possible to calculate the average size of the gene in nucleotide units and this calculation has been performed several times in the past (Pontecorvo, 1958). Taking the average molecular weight of an amino acid as 135 and the average subunit as approximately 20,000, there are about 150 amino acid residues per protein. If the coding ratio is three, then the average structural gene contains about 450 or 500 nucleotide pairs; or, since there are 10 nucleotide pairs per turn (34Å); in the Watson-Crick structure the average structural gene should extend 1,700 Å when stretched out full length.

### *D. Evidence for Colinearity*

The evidence that the amino acid sequence of the tryptophan synthetase protein is colinear with the sequence of genetic sites is given above. Some evidence for the identification of the sequence of genes with the sequence of nucleotides was obtained by Kaiser (1962). His experiment takes advantage of the fact that ultrasonic treatment of DNA shears the molecule and reduces the molecular weight to about 500,000, but once this size is reached further treatment has no effect. DNA was prepared from phage λ genetically marked at both ends of a linkage group. Such DNA can be used in transforming studies when added along with intact "helper" phage (Kaiser and Hogness, 1960). Sonic disintegration of the DNA resulted in preparations which retained transforming activity but in which the linkage between the ends of the DNA was broken as the average molecular weight of the preparation dropped. It could therefore be concluded that the linkage group corresponded to the nucleotide chain, since separating the nucleotides separated the markers. A similar demonstration was that of Bodmer (1962) who reported that treatment of multiply marked transforming DNA from *B. subtilis* with deoxyribonuclease could destroy linked transformation more rapidly than it inactivated single transformation events. One can therefore argue on the basis of limited experimental data, that both amino acid sequence and nucleotide sequence reflect the order of genes on the genetic map and that amino acid and nucleotide sequences are, in fact, colinear.

### *E. Gene Function–RNA Synthesis*

DNA serves *in vitro* as a template for the synthesis of an RNA which has a base sequence complementary to the DNA. Both strands of the DNA can serve as templates for this RNA prepared *in vitro*, as shown both by the quantitative extent of complexing and by the fact that the separate strands of the synthetic RNA can form a double stranded complementary structure *in vitro* (Geiduschek et al., 1961, 1962). The base sequence in the enzymatically produced RNA therefore reflects in a complementary way (*i.e.*, with a cytosine for every guanine in DNA, a uracil for every adenine, etc.) the DNA base sequence. It is a common assumption that the complementary RNA produced by the RNA polymerase is identical with the "messenger" RNA postulated from biological experiments (Jacob and Monod, 1961). Such messenger, or mRNA, originates on a genic template and then migrates to the site of the protein forming system. In microorganisms mRNA is unstable and decomposes after serving as a template for one or several rounds of protein synthesis.

It is possible to detect a fraction of RNA formed very soon after the infection of *E. coli* with bacteriophage, which has a base composition characteristic of phage DNA rather than of the host (Volkin and Astrachan, 1957). Fractions of RNA, which turn over rapidly, have also been demonstrated in organisms synthesizing bacteriophage induced enzymes and these fractions have been observed to associate with ribosomes (Brenner et al., 1961a; Gros et al., 1961). These findings, coupled with the demonstration that RNA can direct the incorporation of specific amino acids into protein-like material (see below), support the idea that the immediate gene product is an RNA which carries genetic specificity from nucleus to cytoplasm. This RNA template may be inherently more stable in mammalian cells than it is in the microbial systems that have so far been studied (Reich et al., 1962).

Yet another class of RNA of great importance for the problem of coding (see below) is responsible for the activation of the amino acids and for their transport to the template on which the protein is finally assembled. This soluble RNA(sRNA = transfer RNA = shuttle RNA) has the following characteristics: a) it is complementary to a short sequence of the DNA, b) a relatively small number (of the order of 40) sRNA sites are present on each DNA molecule, and c) the affinity of the sRNA for the DNA of a particular species decreases as the source of the sRNA become more distant in an evolutionary sense from the source of the DNA (Goodman and Rich, 1962). Mammalian sRNA, for example, will not pair with *E. coli* DNA. Soluble RNA has a molecular weight of the order of 25,000 and contains perhaps 80 nucleotides (Berg, 1961). The molecules behave as though there were an organized secondary structure determined by complementary base pairing (McCully and Cantoni, 1962). A certain number of these nucleotides are "unusual" in that they contain extra methyl groups. It appears that these methyl groups are added to the molecule after it has achieved its total size (Fleissner and Borek, 1962). The sRNA molecules combine specifically with different amino acids and determine the insertion of particular amino acids on the mRNA template (see section IV B).

### *F. In vitro Protein Synthesis*

Crick entitles a recent review "The recent excitement in the coding problem" (1963). Notwithstanding some interesting biological experiments, this excitement comes mainly from the demonstration that amino acids can be incorporated into protein in an *in vitro* system and that the nature of the amino acids incorporated is determined by the RNA added to the reaction mixture. The system consists in part of *E. coli* ribosomes

prepared by grinding cells with alumina, centrifuging, washing, dialyzing and finally freezing the product (Nirenberg and Matthaei, 1961). This preparation along with an energy source, a mixture of amino acids, sRNA, and the proper salts will incorporate radioactive amino acids into protein; the incorporation is routinely measured by precipitating and washing the protein and counting the insoluble radioactivity.

Matthaei and Nirenberg (1961) first demonstrated that addition of the deoxyribonuclease to the cell free system affected only the final level of incorporation of amino acid into protein rather than the initial rate, indicating that intact DNA was not a direct reactant in the incorporation system. It was next found that a source of RNA, other than the ribosomes, was required for incorporation. This additional component could be either natural RNA or it could be a synthetic polymer, *i.e.*, made *via* the polynucleotide phosphorylase (see Steiner and Beers, 1961 for directions for RNA synthesis with the enzyme). A great deal of excitement was aroused by the finding that polyuridylic acid led to the "exclusive" formation of polyphenylalanine (small quantities of leucine were also incorporated by the system) since the experiment seemed to indicate an experimental approach to the assignment of combinations of nucleotides for the determination of particular amino acids.

At least two laboratories immediately started work on the problem, utilizing the following statistical approach and basing their interpretation on a nonoverlapping triplet code: The composition of the polymer formed in a polynucleotide phosphorylase reaction is determined by the molar ratios of the nucleoside diphosphates added. The composition is not necessarily equal to the ratio of reactants and it is advisable to determine the ratio of bases in the polynucleotide product. Polymers of different composition are prepared and used as primers in the amino acid incorporation reaction. The distribution of radioactivity among the amino acids in the insoluble product formed (identified as protein) is determined and correlated with the assumed distribution of nucleotide triplets in the polymer, on the assumption that base sequence in the polymer is random. For example (Table 2), a polymer was prepared containing uridylic acid and guanylic acid (poly U and poly G) in the ratio (frequency) 0.76 to 0.24 (Matthaei et al., 1962). The ratios of all possible triplets would then be given by expansion of $(U + G)^3 = U^3 + UUG + UGU + GUU + UGG + GUG + GGU + G^3 = (0.76 + 0.24)^3 = .439 + .139 + .139 + .139 + .044 + .044 + .044 + .014$. Setting $U^3$ as the frequency of UUU triplets and assuming that this frequency determines the relative incorporation of phenylalanine (set at 100 per cent) and that only one particular order of a triplet containing two nucleotides codes an amino acid, the relative incorporation of the other amino acids permits their

TABLE 2—*Derivation of Coding Units from Amino Acid Incorporation Data: A Partial List*

| Polymer No. | Frequency of triplets relative to frequency of UUU in the initial mixture | | Amino Acids Incorporated Corresponding to Triplets (as assigned) | % Incorporation Relative to Phenylalanine | Other Amino Acids Incorporated (% in brackets) |
|---|---|---|---|---|---|
| 1 | UUA | 13 | Tyrosine | 13 | Leucine (4.9) |
| | | | Isoleucine | 12 | Cysteine (4.9) |
| | UAA | 2.2 | | | Glycine (4.7) |
| | | | | | Glutamic Acid (1.5) |
| | AAA | 0.3 | | | Alanine (1.9) |
| | | | | | Tryptophan (1.1) |
| 2 | UUC | 157 | Serine | 160 | Isoleucine (1.0) |
| | | | Leucine | 79 | |
| | UCC | 244 | Proline | 285 | |
| | CCC | 382 | | | |
| 3 | UUG | 32 | Valine | 37 | Serine (3.2) |
| | | | Leucine | 36 | Argenine (1.1) |
| | | | Cysteine | 35 | Isoleucine (1.0) |
| | UGG | 10.6 | Tryptophan | 14 | |
| | | | Glycine | 12 | |
| | GGG | 3.4 | | | |
| 4 | UUG | 46.2 | Valine | 29.8 | |
| | | | Leucine | 157 | |
| | | | Cysteine | 5.4 | |
| | UGG | 21.0 | Tryptophan | 1.6 | |
| | | | Glycine | 9.7 | Tyrosine (1.0) |
| | | | | | Isoleucine (5.4) |
| | UUC | 147 | Serine | 170 | Methionine (1.5) |
| | | | Leucine | 157 | Glutamic acid (0.44) |
| | UCC | 218 | Proline | 188 | |
| | CCC | 322 | | | |
| | UGC | 68.1 | Arginine | 49.3 | |
| | | | Alanine | 40.4 | |
| | GGC | 31.7 | | | |
| | GCC | 101 | | | |
| | GGG | 1.0 | | | |

Data from Matthaei, et al. (1962).

assignment to triplets. Thus, this particular polymer led to the incorporation of valine, leucine and cysteine at between 35 and 37 per cent of the molar incorporation of phenylalanine. If UUU is set at 100 per cent, then

UUG, UGU and GUU will each occur at 32 per cent of the UUU (phenylalanine) frequency; the triplets UGG, GUG and GGU will each occur at 10.6 per cent and GGG at 3.4 per cent of the UUU frequency. Since valine, leucine and cysteine are each incorporated at close to the expected frequency for some permutation of the U²G triplet, it is likely that one of the amino acids is determined by UUG, one by UGU and the third by GUU. However, this method, as given, does not permit assignment of particular amino acids to particular orders of triplets.

It now appears that the incorporation of some of the amino acids was missed because of the use of trichloroacetic acid to stop the reactions and to precipitate proteins. Polylysine, for example, is soluble in trichloroacetic acid and may be missed unless tungstic acid is also used as a precipitant (Gardner et al., 1962).

The justification for calculating all the results in terms of triplets is a theoretical one. Given 20 amino acids to be determined by four nucleotides it seems necessary to have at least three elements to provide suffi-

TABLE 3—*Current Codons*

| Amino Acid | Incorporated by Poly-* | Triplets Suggested |
|---|---|---|
| Alanine | CG, UGC | UCG, CCG, CAG |
| Arginine | AG, CG, UCG | UCG, AAG, CGC |
| Asparagine | UA, AC, AUC | UAA, AAC, AUC |
| Aspartic Acid | UAG | UAG |
| Cysteine | UG | GUU |
| Glutamic Acid | AG, UAG | UAG, AAG |
| Glutamine | AC, AG, UAG, UAC | GAG, AAC, AGU, UAC |
| Glycine | UG, AG, GC | UGG, GAG, GCG |
| Histidine | AC, UAC | UAC, ACC |
| Isoleucine | UA, UAC | UUA, AAU, UAC |
| Leucine | U, UA, UC, UG | UUA, UUG, UUC, (UUU), UCC |
| Lysine | A, UA, AC, AG | AAA, UAA |
| Methionine | UAG | UGA |
| Phenylalanine | U, UA, UC, UG | UUU, UUC |
| Proline | C, UC AC, CG | CCC, UCC, ACC, CCG |
| Serine | UC, UCG | UUC, UCG, UCC |
| Threonine | AC, CG, UAC | CAC, CAA, CCG, UAC |
| Tryptophan | UG | UGG |
| Tyrosine | UA | AUU, UAC |
| Valine | UG | UUG |

References: Matthaei and Nirenberg (1962); Gardner et al. (1962); Speyer et al. (1962); Jones and Nirenberg (1962); Wahba et al. (1963 a, b).

* Amino acids incorporated by the *in vitro* system in the presence of particular synthetic polynucleotides.

cient variation. This problem will be discussed in greater detail below but it can immediately be seen that a triplet code gives too many combinations, *i.e.*, $4^3$=64. There are 64 − 20 = 44 combinations left over and either these triplets determine amino acids, in which case the code is *degenerate* and more than one triplet corresponds to one amino acid (a possibility neglected in the calculations above), or there are many triplets which have no meaning. The existence of many such nonsense triplets would mean that the genetic material was not efficiently utilized as a source of information. If the code is degenerate then polymers of different composition should lead to incorporation of the same amino acid and this effect has been observed.

The working out of a consistent assignment of triplets has required data from a series of polymers. At first it appeared that all of the coding units contained uridylic acid and a number of workers commented on the possible significance of this finding and based codes upon it (Smith, 1962). It turns out that polymers not containing uridylic acid will permit amino acid incorporation (Gardner et al., 1962, Jones and Nirenberg, 1962) and the speculations on the ubiquity of uridylic acid are therefore unnecessary. The code is probably quite degenerate. A current listing of possible codons is given in Table 3.

## III. Some Problems

### A. *Do Organisms Contain too Much DNA?*

The demonstration that DNA carries structural information for protein synthesis does not imply that all the DNA carries such information. (Structural information determines the sequence of amino acids in peptides as opposed to instructions as to how much or when to produce any protein or group of proteins.) In fact it can be argued that most organisms, particularly more differentiated organisms, contain far too much DNA. The following calculation and tabulation of data (Table 4) may be instructive.

From analytical values for the average amount of DNA per nucleus and from information on the chromosomal constitution it is possible to calculate the average number of nucleotide pairs per nucleus. Assuming that the average number of nucleotide pairs per structural gene is 500 (see above) then phage T4 has sufficient information to code over 300 peptides (some of these would be enzymes required to make phage constituents but not themselves appearing in the completed virus), *E. coli* over 16,000, and the mouse and man well over 8,000,000 (Table 4). Con-

TABLE 4—*DNA Content of Organisms*

| Organism | Nucleotide pairs per organism *or* per nucleus | Triplets[1] | Peptides Coded[2] |
|---|---|---|---|
| Bacteriophage T4 | $2x10^5$ | $6.7x10^4$ | 335 |
| PPLO[3] | $3x10^5$ | $10^5$ | 500 |
| *E. coli* | $1x10^7$ | $3.3x10^6$ | 16,500 |
| Aspergillus | $4x10^7$ | $1.3x10^7$ | 66,500 |
| Drosophila | $8x10^7$ | $2.7x10^7$ | 135,000 |
| Mouse | $5x10^9$ | $1.7x10^9$ | 8,500,000 |
| Maize | $7x10^9$ | $2.3x10^9$ | 11,500,000 |
| Man | $7x10^9$ | $2.3x10^9$ | 11,500,000 |

Data from Pontecorvo (1958) except as noted:

1. Assuming 3 nucleotide *pairs* per triplet.
2. Assuming an average peptide of 200 amino acids or 27,000 molecular weight.
3. PPLO data from Morowitz et al. (1962) in which an average molecular weight of $205 \times 10^6$ per clone forming unit was given for DNA.
4. Value for DNA content of human cells from Leslie (1955). Calculation on the basis of a DNA content of brain cells of 0.68 picograms of DNA-P per cell.

sidering the number of known enzyme reactions and the chemical components and structural proteins in the cell, it seems likely that there are no more than a few thousand different enzymes and/or structural proteins. Krebs and Kornberg (1957) have calculated, for example, that there are no more than about 30 different stages in the systems which make energy available to organisms. Enzymes for these energy yielding steps plus a system for synthesizing protein and other structural components would represent minimum structural information required for a free living organism. Certainly it seems unlikely that the mouse is so much more complex than *E. coli* that it requires 8,000,000 additional (different) peptides. It seems simpler to suppose that there is a relatively small number of structural genes and that much of the additional DNA is involved in regulatory functions. However, this "excess" of DNA would permit organisms to engage in the luxury of a nondegenerate code.

There is information which permits an estimate of the relative number of structural genes. Some years ago Atwood and Mukai (1953) determined the number of genes with "irreparable" functions: that is, genes in Neurospora whose function could not be bypassed by the addition of growth substances to the medium. (These workers used the much more delicate test of allowing mutant nuclei to multiply in the cytoplasm of a heterocaryon, arguing that cytoplasm represents the most satisfactory "complete" medium.) It was found that a major portion of the genes in Neuro-

spora have irreparable functions. Today it seems possible to identify these as regulatory, nonstructural genes. The experiments of Atwood and Mukai *(loc. cit.)* therefore indicate that such genes correspond to a major portion of the genetic material.

Not all organisms carry a surfeit of information. Some members of the PPLO group (pleuropneumonia-like organisms) apparently contain only enough DNA to code for so few proteins that one wonders at the demonstrated ability of these organisms to grow *in vitro* (Morowitz et al., 1962). Obviously the number of enzymes absolutely essential for free living existence is relatively small. But this question of the adequacy of small numbers of different proteins for certain free living organisms makes the apparent excess of DNA in higher organisms more apparent.

### *B. The Variations in the Base Composition of DNA*

Not only is there too much DNA in higher organisms, but there is a wide variation in the relative amounts of guanine + cytosine found in different microorganisms (Belozersky and Spirin, 1960; Lanni, 1960). *E. coli* has a G+C content of 50 per cent, *B. subtilis* 42.4 per cent, *Micrococcus lysodeikticus* 72 per cent, *Tetrahymena pyriformis* 26.5 per cent. Since the DNA extracted from microorganisms is particularly homogeneous in contrast to that of higher organisms (Sueoka et al., 1959) these data indicate that species with different G+C contents have practically no molecules of DNA in common. If the code is universal, that is, if all organisms give the same meaning to each triplet, then this G+C variation would seem to indicate a wide divergence in protein composition between the different organisms. Such a wide divergence is not obtained experimentally.

Sueoka (1961) has attempted to correlate the variations in protein amino acid composition that do exist with G+C content in an effort to determine coding relationships. The different proteins of any one microorganism are quite similar in amino acid composition since the relative protein amino acid composition is the same regardless of the medium on which the organisms are grown. Microorganisms grown on rich medium containing preformed amino acids and other nutrients have a very different complement of enzyme proteins than do the same organisms grown on minimal media where such nutrient substances must be synthesized *de novo;* since repressive mechanisms prevent the synthesis of the synthetic enzymes required to produce amino acids and other "building blocks" when organisms are grown on rich medium. Since large differences in the type of protein produced do not affect the over-all protein amino acid composition it is likely that all the proteins of an organism are

quite similar in composition. One would therefore expect that the relative frequency of penylalanine in the protein of an organism with high G+C content should be independent of growth conditions and lower than the frequency in an organism with a low G+C content, if the coding triplet for phenylalanine is UUU. Such a correlation was obtained (Table 5).

TABLE 5—*Correlation of Protein Amino Acids with DNA Base Content*

| Correlation with G+C Content | | |
|---|---|---|
| Negative | No Correlation | Positive |
| Isoleucine | Histidine | |
| Lysine | Valine | Alanine |
| Aspartic acid and asparagine | Leucine | Arginine |
| Glutamic acid and glutamine | Threonine | Glycine |
| Tyrosine | Serine | Proline |
| Phenylalanine | Methionine (possible) | |

Data of Sueoka (1961).

The following assumptions were made by Sueoka *(loc. cit.)* 1): that all the DNA codes for protein, that is, that there is little or no "non-structural" DNA and 2), that the code is universal. Relatively weak correlations were obtained, indicating that if there is a universal code, it must be highly degenerate. The approach used was sound even if most of the DNA does not code for structural protein, if it is assumed that the base composition of the DNA used for coding the structural information in protein is similar to the over-all base composition. In fact, most of the structural genes of an organism must have similar base composition. To be sure, it has been possible to separate some markers by their differential sensitivity to heat denaturation (Roger and Hotchkiss, 1961; Ginoza and Zimm, 1961), but one might be impressed as much by the closeness of the denaturation temperatures of different markers, which depend on G+C content, as by the fact that they are separable.

The existence of these differences in the base content of different organisms raises questions which are not yet answerable. For example, if all organisms are considered as related by evolution, how has the DNA base composition changed so greatly without causing serious changes in amino acid content? Some of the questions involved in the evolution of the different base sequences have been considered by Freese (1962).

### C. *Self-Reproducing RNA*

The demonstration that RNA isolated from plant (Gierer and Schramm, 1956) and animal viruses (Alexander et al., 1958) is infectious along with the elegant demonstration that mutant RNA's determine protein with altered amino acid sequence (see Tsugita and Fraenkel-Conrat, 1962) is adequate evidence that RNA codes for protein. The discovery of an RNA-containing bacteriophage (Loeb and Zinder, 1961) only reinforces the impression of the wide distribution of self-replicating RNA carrying structural information. How this RNA differs from that found in the ribosomes or from messenger RNA is not known: one seems stable and self-replicating, the other unstable (in microorganisms) and unable to replicate. Consider the case of a plant infected with a systemic virus: even though the necessary enzyme systems are present one type of RNA replicates and the other does not, and we are unable to determine where the structural difference lies. Both types of RNA carry structural information, both attach to ribosomes (Barondes and Nirenberg, 1962; Haselkorn et al., 1963); both would appear to have similar structure but with different biological properties. The paradox is worth emphasizing if only because we have been aware of it for some years.*

## IV. The Genetic Code

### A. *General Nature*

The nucleotide sequence of the genetic material is related in a colinear way to the amino acid sequence of protein. It is now required that we suggest in a more definite way the relationship between particular bases and particular amino acids. We require that the exact sequence of bases corresponding to each amino acid be determined and we require that the absolute linear direction of these be indicated to specify the structure of the gene from its starting point. In fact, we require some evidence that the gene has a unique starting point.

It is assumed that the gene is read by the enzymatic mechanisms which lead to the synthesis of complementary RNA.

There are four naturally occurring bases (or substitutes for these, considering the 5-hydroxymethylcytosine and the 5-hydroxymethyluracil

* *Note added in proof:* It now appears that viral RNA carries instructions which cause the host to synthesize a virus specific RNA polymerase (Baltimore et al., 1963). This enzyme is not formed normally in the cell and cellular RNA synthesis is DNA dependent. Once the RNA dependent polymerase is formed viral RNA, which induced its formation, becomes self-replicating.

[Kallen, et al., 1962] found in certain bacteriophage as a substitute for thymine) in the DNA and 20 amino acids to be specified. It is argued that the disulfide amino acid, cystine, is not directly coded, but rather results from oxidation following the structural juxtaposition of cysteine residues and that hydroxyproline is probably formed from proline *in situ*. Taken one or two at a time there are not enough combinations to determine the 20 amino acids; a code of two elements would have $4^2=16$ possible permutations. Since there are 20 items to be determined it is evident that at least some of the items must be determined by three bases. Since uniformity is the simplest possibility, a coding element of three bases per amino acid has generally been suggested as a basis for possible codes simulating nature.

No definitive evidence exists to specify the coding unit as three (or even a multiple of three) and there is no evidence which requires a uniform number of symbols per coding unit. Perhaps the real reason for disregarding the possibility of codes with a varying number of symbols is the finding that single base substitutions, but not additions or deletions, lead to proteins containing substituted amino acids (Tsugita and Fraenkel-Conrat, 1962). If the observed replacements were strictly random so that any amino acid could be inserted as a result of some single base substitution, then a variable number of units per codon would be impossible. The data on substitutions do not indicate that amino acid substitutions are random (Woese, 1961; Ycas, 1962); some substitutions seem to occur more frequently than would be expected by chance. In part, I believe, this is a consequence of the "fingerprint technique" used for detecting changes in proteins and in amino acid sequence. Preliminary screening of hemoglobin proteins for the detection of mutants to analyze requires a preliminary electrophoretic separation. Only amino acid substitutions which alter the charge properties of the peptides are easily detected, thereby restricting the changes likely to be found, *i.e.*, substitution of an amino acid by another of like charge might not be detected (Crick, 1963).

The following biological evidence indicates that the code is either based on triplets or on a multiple of three bases per codon. Crick et al. (1961) have obtained a series of mutants of bacteriophage T4 which can be interpreted (see below) as due to additions or deletions of single base pairs in the DNA. It is possible to classify mutants arbitrarily as additions or deletions by picking one as a standard and referring all others to that standard. Genetic combination of an addition with a deletion not too far away on the genetic map partially restores genetic function. Genetic combination of two additions or two deletions results in an inactive genome but combination of three additions of three deletions can result in an

active genome. If reading of the gene occurs in groups of three bases, then addition or deletion of one or two elements would be expected to throw the reading mechanism completely out of phase. All of the amino acids beyond the point of the addition or deletion (of one or two bases) would be coded by changed triplets (except for those few which by chance would still have a meaningful triplet) and the protein resulting, if one is formed at all, would be so completely changed as to be without any vestige of its original function. Movement of the "carriage" by three units, however, would restore the reading frame and permit most of the amino acid sequence to be normal, although there might be an addition or deletion of a single amino acid in the peptide. The hypothesis of codon triplets therefore accounts for the data. In fact, these findings are as good evidence as any that many spontaneous and acridine-induced bacteriophage mutants contain additions or deletions. It is a necessary assumption of the above argument that only a major portion, but not all, of the protein structure is required for function and there is good evidence for this assumption (Braunitzer et al., 1961; Freese, 1962).

The sequence ABCDAB can be thought of as having either two (ABC, DAB) or four (ABC, BCD, CDA, DAB) triplets. A reading mechanism that sees four triplets is characteristic of an overlapping code; a mechanism recognizing only two triplets is of the nonoverlapping type. Any simple overlapping code of the type indicated above is eliminated by the random arrangements of amino acids in peptides (Brenner, 1957; Ycas, 1958) and by the finding that nitrous acid induced mutants of tobacco mosaic virus can have single amino acid substitutions in the coat protein. Changes in what seems to be a single base produce only a single amino acid substitution, contrary to the expectation of the completely overlapping code indicated above. Present data do not eliminate all overlapping codes. Wall (1962) shows that a code in which there is a single overlap and in which the code word length is 4 (*i.e.*, ABCDABCDABCD with code words of ABCD, DABC, CDAB) could account for the data, including those of Crick et al. (1961). A partially overlapping code with an even number of units accounts for the finding of Sueoka (1961) that the proportion of certain amino acids is not correlated with the G+C content (Table 5) and, in fact, Sueoka did suggest that a four unit code would be particularly convenient. The findings with the *in vitro* system are beside the point. It would be possible to account for the amino acid incorporations by models other than the triplet model used. In fact, it must be admitted that analysis of the data in terms of a triplet, nonoverlapping code is not *required* by any of the present data but is, rather, a convenient interpretation.

Several investigators (Roberts 1962 a,b; Jones and Nirenberg 1962;

Wahba et al., 1963) have commented on the finding that 18 of the 20 amino acids can be incorporated *in vitro* using synthetic polynucleotides containing only two bases (Table 3). Roberts (1962 a,b) has proposed a two element code with no degeneracy, accounting for the ambiguities such a code would introduce by supposing either that the code is mixed, containing a few three letter symbols, or that "additional information" will some day account for the apparent inconsistencies of such a scheme. It is evident that the data obtained with the *in vitro* system do not seem to *require* triplet elements for all amino acids and it is therefore likely that other alternative and "mixed" schemes will be proposed until sufficient amino acid substitution data (section VII) is accumulated to uniquely specify the code.

### *B. Degeneracy and Its Biological Significance*

If there are 64 triplets coding for 20 amino acids then either some of the triplets do not code for amino acids, or more than one group of three bases must indicate the same amino acid. The latter is the case for a degenerate code. Evidence for degeneracy of the code comes first from studies with the synthetic system in which it has been determined that more than one combination of bases can code for a particular amino acid (see above, section II F). The most convincing results have to do with the nature of sRNA.

The insertion of an amino acid into its place on a growing peptide is a function of the specificity of the sRNA molecule to which it is attached (Chapeville et al., 1962). The copolymer of uridylic and guanylic acid will incorporate cysteine but not alanine into protein and this incorporation requires a specific sRNA. If cysteinyl-sRNA is prepared and then treated with Raney nickel it is converted into alanyl-sRNA and this material incorporates alanine into protein on the poly UG template. Alanine is not incorporated by the *in vitro* system primed by the UG copolymer when alanyl-sRNA is formed with the sRNA specific for alanine. Therefore, it is the RNA portion of the amino acid-sRNA complex which recognizes a particular site on the polynucleotide template.

If it could be shown that there was more than one sRNA specific for a given amino acid then there would be a chemical basis for supposing the code was degenerate. This sort of evidence was provided by Weisblum et al. (1962) who showed that there were two leucine-acceptor RNA's separable by counter current distribution. The incorporation of leucine into protein when leucine was attached to sRNA was stimulated by poly UC for one of the RNA's and by poly UG for the other.

This evidence does not indicate the degree of degeneracy; that is, one

might find only 30 of the 64 triplets specifying amino acids or, contrariwise all 64 might specify amino acids. Offhand, it seems unlikely that all nucleotide sequences specify some sequence of amino acids, if only because this implies that *all* gene functions must be expressed via protein. If, for example (Goodman and Rich, 1962), sRNA is constructed directly off a DNA template there must be something which prevents this RNA from serving as a template for peptide synthesis. If all nucleotide sequences coded for amino acids, all the regulatory functions of genes would also have to be mediated via protein. The most widely quoted evidence suggesting a high degree of degeneracy is the observation that the over-all amino acid composition of microorganisms does not vary very much even though the perecentage of G+:C in the DNA may differ by 50 per cent (*i.e.*, from 25 to about 75 per cent G+:C, see section III B). If the code is degenerate—that is, if the same amino acids can be specified by DNA's high in A and T as by material high in G and C—then the results make sense.

Related to the problem of degeneracy is the question, "Is the genetic code the same in all organisms?" generally put as the question of "Universality" of the code. The common use of the same bases and the same amino acids in organisms seems to suggest that the codes should not be too different. The general applicability of the base triplets worked out with the *E. coli in vitro* system to amino acid substitutions in human hemoglobin, tobacco mosaic virus and the tryptophan synthetase A protein of *E. coli* suggests universality. Protein can be made *in vitro* with *E. coli* ribosomes and sRNA but with templates from different systems. Thus, it is possible to make peptides reminiscent of the coat protein of tobacco mosaic virus and hemoglobin can be made by ribosomes of the rabbit using *E. coli* sRNA (von Ehrenstein and Lipmann, 1961). Poly U leads to the formation of polyphenylalanine in systems derived from mammals as well as from *E. coli* (Maxwell, 1962; Weinstein and Schechter, 1962). For the biochemist, demonstration that a coding element is the same in *E. coli* and the rat is equivalent to the demonstration that the code is universal. It probably is.

### *C. Problems of Reading*

Any message has punctuation marks, a place where the message begins and a direction in which it is read. Not all languages read in the same direction and some read in more than one. Japanese still reads up and down from right to left in some books, and horizontally from right to left in others. Books read up and down ordinarily are read from back to front,

books read horizontally ordinarily read from front to back. Notwithstanding this complexity there is a minimum of confusion (if one can read). A code could probably be constructed which read one way in one gene and in the opposite direction in another. However, it is reasonable to ask whether the code is, in fact, such that any message must always be read in the same direction. The question cannot be answered at present for a whole linkage group.

The complementary two-stranded nature of the DNA molecule is an additional problem. Poly U codes for polyphenylalanine and it seems as though poly A codes for lysine (Gardner et al., 1962; Jones and Nirenberg, 1962). If the sequence TTT occurred in one strand of the DNA the sequence AAA would occur in the corresponding strand. Now *in vitro* experiments clearly indicate that both strands of the DNA can act as templates for complementary RNA production, since the RNA strands produced are themselves complementary (Geiduschek et al., 1962). If both strands were separately active *in vivo* then each would produce a different protein if both were read in the same direction. Alternately, either the A:T base pair would have to be read as a unit with the pair specifying one or the other of the amino acids or, as now seems likely, only one of the strands of the DNA may be active for protein synthesis.

The best evidence for this idea comes from the experiments of Champe and Benzer (1962). Mutants of bacteriophage T4 unable to grow on *E. coli* strain K12 were obtained by methods which ensured that the change was due to the conversion of a G:C to an A:T pair. When such mutants were used to infect K12 bacteria in the presence of 5-fluorouracil about half of them grew and produced progeny (A:T to G:C mutants do not respond to fluorouracil). The effect was not due to mutation since the progeny phage were unable to grow on K12 in the absence of fluorouracil. It is supposed that fluorouracil is incorporated into mutant messenger RNA instead of uracil. In a proportion of the cases the analog mistakenly pairs with guanine on an amino acid acceptor (sRNA) molecule. This must be considered a double mistake. The original mutation changes the template so that it recognizes sRNA carrying an "incorrect" amino acid into the protein specified by the mutant gene. The presence of fluorouracil in the mutant m-RNA means that occasionally this mRNA will make a mistake and pair with sRNA carrying the *original* amino acid. The result of both mistakes would be the production of some normal protein. Since on a random basis one would expect half of the mutants to be A:T at the mutant site while the others are T:A and since on the average only half of the mutants respond to fluorouracil, Champe and Benzer conclude that only one of the DNA strands is functional, and that the fluorouracil is effective only when there happens to be an A on the DNA strand

which is read, or rather on the DNA strand which is physiologically active.*

The first ideas of macromolecular synthesis on a template now seem somewhat naïve since it was supposed that the demonstration that these substances were formed without the intervention of free intermediates (Spiegelman, 1957) required that the components all lined up more or less simultaneously on a template and that they were then "zipped together." It was therefore meaningful to ask whether the formation of peptide bonds occurred sequentially or at random throughout what was to be the peptide chain. For hemoglobin at least, the peptide bonds are formed in sequence starting from the amino terminal end and proceeding toward the carboxyl end (Dintzis, 1961; Naughton and Dintzis, 1962). At the same time it is becoming evident that only one amino acid-sRNA complex at a time attaches to the ribosome on which the growing peptide chain occurs (Hoagland and Comly, 1960); a concept which makes sequential synthesis necessary. The same type of finding seems to be indicated for biosynthesis of the DNA polymer. Although the requirement for denatured DNA to serve as a primer in the mammalian DNA polymerase (Bollum, 1959) and in the T2 DNA polymerase (Aposhian and Kornberg, 1962) seems to require that the DNA split into two portions along which synthesis might proceed in either direction, there is no evidence for a single stranded DNA inside a cell (even the single stranded øX174 phage goes into a two stranded stage as soon as it enters the host) and it seems likely that DNA synthesis proceeds simultaneously with unwinding. Cairns (1963) has actually obtained autoradiographs of DNA replication in *E. coli* which fit this picture and Yoshikawa and Sueoka (1963) and Nagata (1963) have reported experiments which show the sequential synthesis of markers in *B. subtilis* and in *E. coli*.

There is some genetic evidence that the DNA is read sequentially for purposes of specifying the phenotype, that is, for the synthesis of messenger RNA. This interpretation of the experiments requires the assumption that the mutagen acridine acts by causing additions or deletions of base pairs to the DNA. As indicated above, two types of mutants of the T4 bacteriophage can be obtained and these can be classified as (+) or (−) by reference to a standard type (Crick et al., 1961). When (+) and (−) mutants are combined, gene function is partly restored and Crick interprets the phenomenon as follows: the gene is read sequentially in triplets starting from a fixed point. Addition or deletion of a single base

* *Note added in proof:* Evidence has now been obtained in a number of laboratories (Spiegelman, Hall, Marmur, Cold Spring Harbor Symposium 1963, in press) that messenger RNA formed *in vivo* will form a complementary hybrid with only one of the two DNA strands.

throws off the reading of all succeeding groups of three bases. Much of the sense of the protein can be restored by a second mutation of opposite nature (deletion for an addition) which restores the reading frame and which will, if its site is not too far from the original mutation, lead to a protein without too many changes and this protein may be functional. This argument works only if the gene is read sequentially, otherwise one would not expect the triplets in other portions of the molecule to be misread.

When these experiments were originally reported, Crick supposed that the code was without "commas," that is, without some structural arrangement such as a particular base which divided the messenger RNA into particular codons. He has now (1963) changed his mind and supposes that the commaless code is highly unlikely because it would not account for the results with the *in vitro* system. For example (Levinthal, 1959), the sequence UUUUUU would be ambiguous since the UUU sequence appears in an overlap and an amino acid position could not be specified. It would seem that the requirement for sequential reading of the messenger, starting at one end with only one sRNA adapter attached to the messenger-ribosome-growing peptide at one time, makes this question superfluous.

An additional bit of evidence comes from those deletions in which a portion of each of two adjacent genes (rIIA and rIIB) in phage T4 is removed. Ordinarily the two genes function independently but in the deletion mutant both are affected by the presence of either a (+) or a (−) mutation in what must be considered the first (in a sequential sense) of these genes. The interpretation is that the deletion, by removing the starting point of the second gene, ties it to the reading of the first.

This type of reasoning leads to conclusions quite similar to those reached by Jacob and Monod (1961) and which forced them to suppose that groups of structural genes are regulated by a locus, called the operator, at one portion of the gene and that it is at this point that the reading of structural information starts. Regulation of gene function is presumed to occur by combination of repressor type substances with this operator thereby preventing transcription of the genetic information.

### *D. Conclusion*

It is possible that the genetic code is one of nonoverlapping triplets read in one direction and that the code is highly degenerate. Only one of the two strands of DNA is "read" for protein synthesis but we have no idea how the choice of which strand is read is made. These ideas are not uniquely required by the data available.

## V. Method for Changing Base Order with Chemical Mutagens

These are two general methods by which the details of the sequence and number of bases required to specify a particular amino acid can be determined. The first method uses as template material in the synthetic system polynucleotides prepared by methods which specify the sequence. Analysis of the peptides produced on such a template gives immediate information about the details of the code. Polymers made by priming the polynucleotide phosphorylase with small quantities of AU and larger quantities of uridine diphosphate promoted the incorporation of tyrosine and phenylalanine but not isoleucine into protein. In contrast, poly UA made with uridine diphosphate and adenosine diphosphate stimulated the incorporation of tyrosine and isoleucine to the same extent. Some of the tyrosine was found at the carboxyl end of the peptide. These results are taken to indicate (Wahba et al., 1962) that the coding unit for tyrosine is AUU. Since it is still difficult to prepare and analyze ordered sequences of nucleotides progess using the approach may not be as rapid as it was with the unordered polymers.

A second method is based on the analysis of changes of amino acid sequence resulting from mutations that have altered single known base pairs. If different mutations can be obtained affecting the same codon, and if the relationships between these mutations can be ascertained by recombination analysis, then it should be possible to make some definite statements about the nature of the code. If sufficient changes become available it will be possible to eliminate certain types of code (Levinthal, 1959). Analysis of amino acid replacements itself, without genetic data, can be very useful (see section VII) but it is the combination which gives most information.

### A. *Possible Changes in Base Sequence*

If genetic information is coded in the sequence of bases in a DNA made up of a Watson-Crick double helix, then any change in the sequence must result in either mutation or inactivation of the gene. Any change which permits the DNA to reproduce is a mutation but since the concept of mutation requires heritability, inactivations which prevent DNA replication cannot be so classified in any organism in which failure of a DNA molecule to replicate leads to failure of the whole organism to reproduce, as in viruses and bacteria. Freese (1959) has classified the possible types of genetic alteration as follows: Substitution of a purine for a purine or a pyrimidine for a pyrimidine is called a transition; A:T to G:C, G:C to A:T, T:A to C:G and C:G to T:A represent possible transitions. Substi-

tution of a pyrimidine for a purine or a purine for a pyrimidine is a transversion; A:T to T:A or C:G, G:C to C:G or T:A, T:A to A:T or G:C and C:G to G:C or A:T are possible transversions. It is supposed that it is possible to extend the DNA chain by one or two base pairs or to delete one or two by certain treatments (Brenner et al., 1961b). The transfer and inversion of larger segments of genetic material is well known in genetics but it will not be considered here.

What is pertinent to studies on the genetic code is the means by which particular changes can be induced and the certainty with which it can be stated that a specified change has been induced. For example, the compound ethyl methanesulfonate is a potent mutagen and reacts primarily with guanine to cause transitional change from a G:C to an A:T pair. Occasionally the transition may be A:T to G:C. Suppose an ethyl methanesulfonate induced mutation results in substitution of a particular amino acid at some site (Rothman, 1961). How can one be sure which base change has occurred in this particular instance? If treatment with an agent inducing mainly A:T to G:C transition caused reversion to the *original* genotype, then the primary change would be specified.

### *B. General Requirements for the Study of Mutation*

Evaluation of such tests requires description of the techniques used to determine the site and nature of mutations, particularly back mutations. In those cases in which bacteria are used and an enzyme activity is affected (particularly when a change in activity is reflected as a change in nutritional requirement) the level of activity of the enzyme in question is the primary test. "Reverse" mutations to restoration of activity are scored in the first instance by the loss of nutritional requirement or by the appearance of enzymatic activity. When a test for back mutation (return to enzyme function) is made it is characteristically found that the second step mutants are of many types. One observable class (when microorganisms are considered) behaves like the original in terms of colony size or growth rate. Other classes of lower growth rate or enzyme content can also be observed. These classes of small colony mutants are different from the original and can represent several different types of genetic change (Fig. 2). They may represent double mutants containing *both* the original change and a genetically unlinked second change, the combination of the two mutations giving a partially restored phenotype. Such unlinked *suppressor* type mutations may suppress any mutation within a gene or they may be allele specific, that is, suppress only certain changes in certain codons. It is these last which are of most pertinence to the problem of the genetic code (Benzer and Champe, 1961). Another class of secondary mutation,

much more common than was thought possible, is due to changes in the *original* gene other than at the site of the primary mutation and separable from it by genetic recombination. One class of these mutations restores gene function by introducing a second substitution into the mutant protein (Rothman, 1961; Garen, 1961). Such intragenic suppressors operate when both primary and secondary base changes result in sequences which still code for some amino acid and where a protein is still constructed. How it is that mutant proteins may be restored to partial function by secondary amino acid substitutions is a problem for protein chemists. Still another type of intragenic suppressor has been indicated in the discussion above. Mutations which appear to cause single base additions or deletions can be suppressed by compensating additions or deletions as suggested by Crick and his co-workers (1961). Suppressors closely linked to the site of the original mutation are known in bacteria but it is only in the bacteriophage T4 that a system working by additions and deletions has been seriously suggested.

Secondary mutations which result in only partial reversion of the organism to the same phenotype can occur *within the same codon* (Fig. 2). Such mutations may be of two types: a) mutations recombining with the

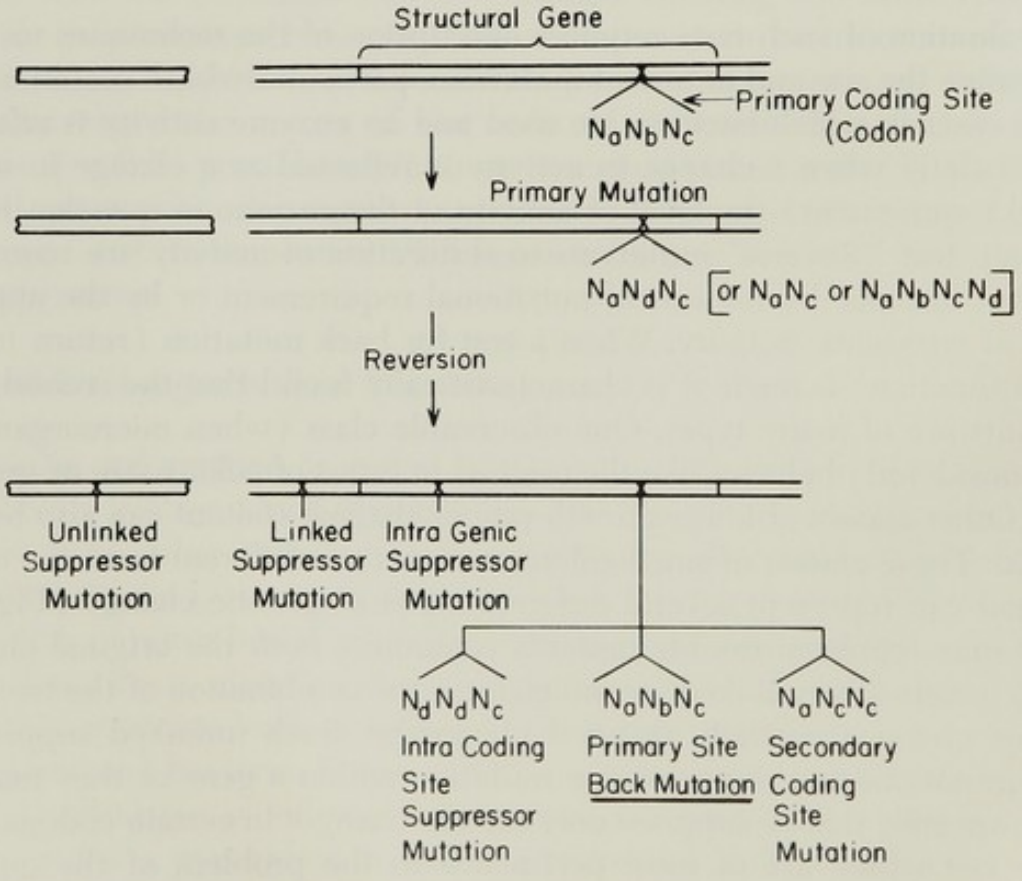


FIG. 2—Types of genetic change leading to "reversion."

original mutant at extremely low frequencies in which it seems that a nucleotide pair adjacent to that originally affected has been changed, and b) those which will not recombine with the original, indicating that the originally affected base pair has been changed but not necessarily back to the original. "Back mutations" to what appears the full wild type phenotype have been obtained which yield proteins with amino acids different from the original but which function with wild-type efficiencies. Such proteins may result from changes at either the original or at adjacent nucleotides (Henning and Yanofsky, 1962). What these findings imply is that it is not possible to determine mutagen specificity by looking at the gross phenotype of revertants; the revertants must be analyzed genetically to see whether they represent reversion at the original locus. In fact, it is not even possible to call any particular mutation a back mutation without both adequate genetic studies and a complete amino acid sequence analysis of the protein product to be certain that mutation is truly a reverse mutation to the original configuration. It is unfortunate that these recent discoveries make many of the previous studies on mutation (including a number of the reviewer's) ambiguous, since it is less certain than ever just what was being measured. Luckily, the phenomenon of mutagen specifiicity does make it possible to use the mutation phenomenon as a source of information about the code if care is used.

### *C. Transitions Produced by Base Analogs*

When bacteriophage T4 is used to infect cells in a medium in which thymine synthesis is suppressed and to which bromouracil has been added, the bromouracil is incorporated into the phage DNA with a concomitant increase in the frequency of mutations (Litman and Pardee, 1956, 1960). 2-Aminopurine is also incorporated into phage DNA with mutagenic consequences. The compounds bromouracil and 2-aminopurine are mutagenic for bacteria when the culture medium is properly designed to promote incorporation (Rudner, 1961). Bromouracil incorporated into mammalian cells increases the frequency with which chromosome breaks are observed (Hsu and Somers, 1961). These compounds induce transitions: if bacteriophage *r* mutants are induced in the first instance by aminopurine or bromouracil then such mutants can be induced to revert by a second stage growth cycle with bromouracil. If, on the other hand, spontaneous mutations are collected or if mutations are induced with proflavin then most of these mutations are not induced to revert to the wild phenotype by bromouracil (Freese, 1959). This experiment shows that there is some difference in the type of mutation induced by bromouracil or aminopurine on the one hand and by pro-

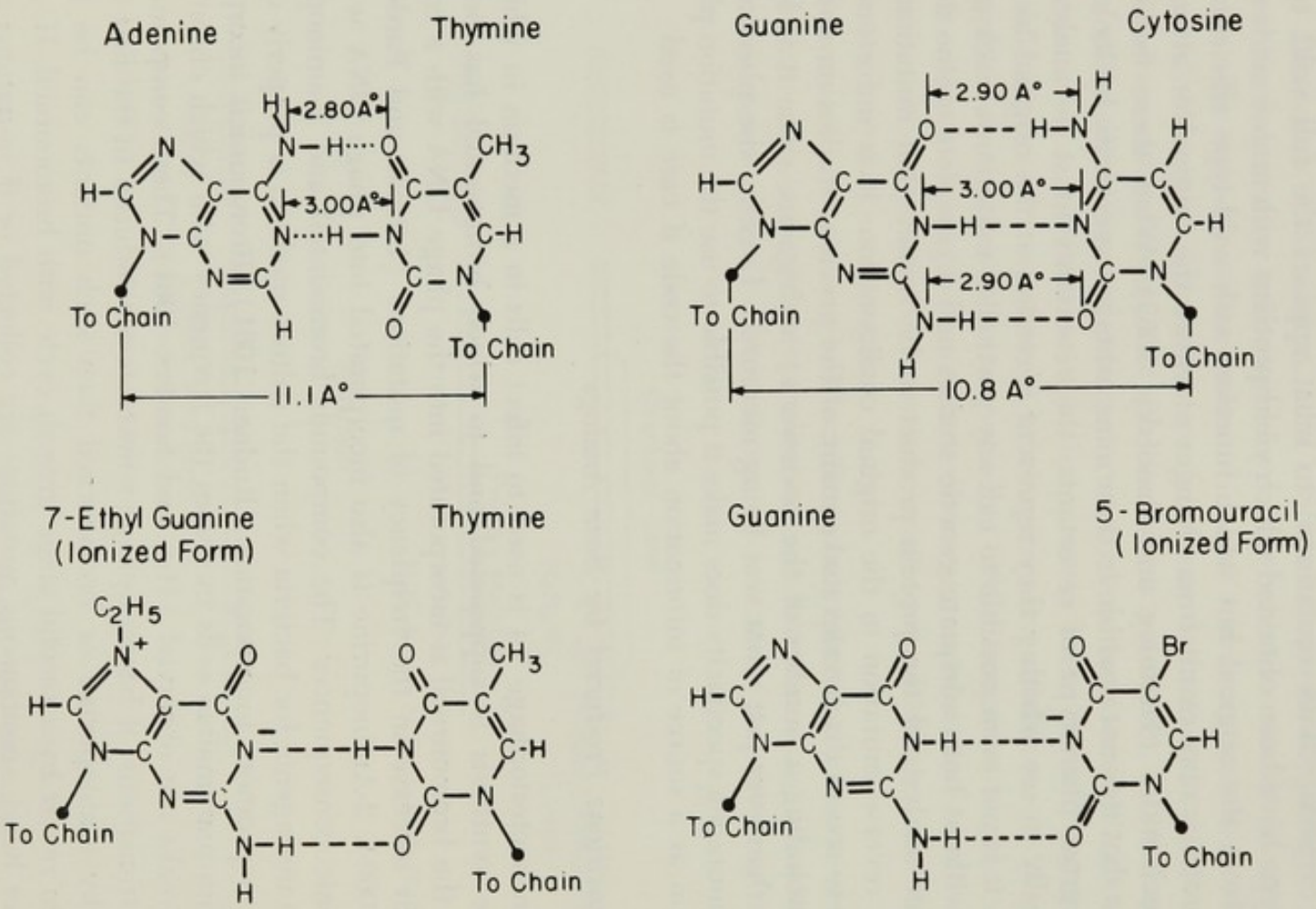


FIG. 3—Normal and abnormal base pairings in the Watson-Crick structure (dimensions after Pauling and Corey, 1956).

flavine and for (most) spontaneous mutations on the other. On structural grounds it is likely that both bromouracil and aminopurine should lead to mispairings at replication; such mispairings should lead to the altered base sequences which are mutations.

In the Watson-Crick structure for DNA the specificity of pairing, guanine with cytosine, adenine with thymine, depends on the selection of particular tautomeric forms. If other tautomeric forms are selected thymine will pair with guanine. Any additional substituent on any nucleotide base may change the relative proportion of the time an unusual tautomeric form is to be encountered, depending on the chemistry of the substituent. Bromouracil is an analog of thymine since the Br group has about the same radius as the $CH_3$ group at the 5 position of thymine. It does not have the same inductive effect as the $CH_3$ and therefore is likely to increase the percentage of times odd tautomeric forms will occur (Freese, 1959). An alternative interpretation is that changes in the pK caused by introduction of the Br atom lead to altered ionized forms which pair with guanine (Fig. 3). (Lawley and Brookes, 1961). This increases the percentage of the time thymine will pair with guanine rather than with adenine and hence increases the mutation frequency. 2-Aminopurine is an analog of adenine (6-aminopurine) and may be incorporated instead of the adenine. Even without tautomeric change 2-aminopurine can pair with cytosine. Aminopurine is incorporated into the DNA to a relatively slight extent compared to bromouracil, but it is so efficient in causing mispairing errors that the frequency with which mutations are induced is relatively high.

Bromouracil may cause either the A:T to G:C transition or the reverse. When incorporated into the DNA it may cause errors at replication as follows: BU:A gives the pair BU:G. On further replication the G pairs with C to give C:G. However, if bromouracil transformed into bromodeoxyuridine triphosphate makes a mistake at replication so that it pairs with guanine in the DNA template we have G:C giving G:BU which on further replication gives in the majority of cases A:BU and then one step further A:T for the G:C to A:T transition. Both these different types of action have been differentiated experimentally (Terzaghi et al., 1962).

Champe and Benzer (1962) suggest the following rule, "Given a mutant (of any origin), if it is inducible to revert by 2-aminopurine, it can be classed as a transition mutant. If it reverts strongly in response to bromodeoxyuridine (equivalent to bromouracil) and hydroxylamine as well (a mutagen presumed to act by reaction with cytosine), this suggests that the mutant pair is G:C; if it does not the mutant base pair indicated is A:T."

It is my intention to examine in some detail the specificity of the muta-

gen ethyl methanesulfonate and two other mutagens to see how well and with what confidence these rules can be applied to deduce changes in the code.

## VI. The Action of Particular Mutagens

### A. *Alkylating Agents*

Ethyl methanesulfonate, $C_2H_5OSO_2CH_3$ (EMS), has been much used as a mutagen because of its high efficiency and low toxicity. It was the first mutagenic substance to show activity on mature bacteriophage (Loveless, 1959); mutagens like aminopurine and bromouracil work only on vegetative phage. The methyl methanesulfonate (MMS) analog is mutagenic for bacteria (Strauss, 1961) but is not mutagenic when used to treat mature phage. Both methyl and ethyl methanesulfonate inactivate bacteriophage by what appears to be a two stage reaction and treatment of organisms with either agent induces a decay reaction which continues after removal of the mutagen (Loveless, 1959).

According to Brookes and Lawley (1961) the site of reaction of low concentrations of EMS with DNA is at the 7 position of the guanine residues. Alkylation of guanine in DNA results in the formation of a quaternary nitrogen. The distribution of this charge over the purine ring unstabilizes the bond of purine to deoxyribose and leads to the splitting off of alkylguanine, leaving an apurinic acid residue. In contrast to DNA, alkylated ribonucleic acid is stable in aqueous neutral solution. Apurinic acids are less stable than those with a full complement of purines and the DNA chain is subject to hydrolytic scission following depurination.

Two hypotheses have been suggested to account for the mutagenicity of EMS. The first (Bautz and Freese, 1960) supposes that DNA with apurinic acid sites can still reproduce; the apurinic sites do not determine any particular complementary base at replication and as a result there is a random filling of the opposite position. Since the chemical results suggest that guanine is the major reaction site the sequence of events would then be: G:C to O:C (where O represents the apurinic site), on replication O could pair with either A, T, G or C. On further replication one would obtain one of the following: G:C representing no change, T:A and C:G representing transversions and A:T representing a transition. The prediction would be made that the transition mutant should not revert as a result of treatment with EMS but that one class of transversion mutant should revert with EMS. Pal (1961) has reported the alkylation of free adenine by ethyl methanesulfonate (EMS). Reaction of adenine might

result in the A:T to G:C transition as well as in the other changes but, as suggested by the chemical results, at much lower frequencies. This hypothesis also predicts that there should be an increase in mutant frequency with time of storage (of organisms or DNA) after removal of mutagen, since the second stage reaction of depurination is required for mutagenesis.

The second hypothesis was suggested by the finding (Lawley and Brookes, 1961) that addition of an alkyl group to the 7 position of a guanine with its 9 position substituted already changed the ionization constant of one of the ring nitrogens involved in hydrogen bond pairing. This change in pK could then lead to a change in the ability of the alkyl guanine to hydrogen bond with cytosine and would make pairing with thymine more likely (Fig. 3). All EMS mutations should then be G:C to A:T transitions except for a small fraction of A:T to G:C transitions. The induced mutations should not be induced to revert with EMS or at best should revert with low frequency because of the predilection of EMS for reaction with guanine.

Most of the EMS induced mutations observed in bacteria behave like transitions. Balbinder (1962) observed that 16 out of 23 tryptophan-requiring mutants reverted upon treatment with diethyl sulfate (an ethylating agent similar in alkylating action to EMS) and upon treatment with one or the other of the base analogs; of the remainder four would not revert upon treatment with any mutagen. In this laboratory (Schwartz, 1963), 38 EMS induced mutants of *E. coli* were obtained and tested for their reversion patterns. The test system permitted rough phenotypic screening of revertants as to genetic type. Constitutive *lac*$^+$ cells were used to produce *lac*$^-$ *(i*$^-$*z*$^-$*)* mutants and these *lac*$^-$ cultures were then treated with mutagen and plated on medium containing filter sterilized lactose as a sole carbon source. Two major types of colonies always appeared; a large type surrounded by a halo of small colonies and colonies lacking this halo. The large colonies had normal (i$^-$z+) levels of $\beta$-galactosidase activity and transducing phage prepared from these strains transduced different *lac*$^-$ alleles. The other type colonies (small) had low $\beta$-galactosidase activity and transducing phage prepared from these organisms transduced only the *lac*$^-$ allele from which the revertants were originally obtained. In recombination analysis the small colony characteristic was transferred as though it was very close to the *lac* gene. Regardless of whether the small colony mutations are interpreted as intra-genic suppressors or partial reversions it is evident that they do not represent mutations back to the original genotype (see Smith-Keary, 1960 for discussion of a similar genetic analysis); whether the large colonies represent true back mutants is a question discussed above.

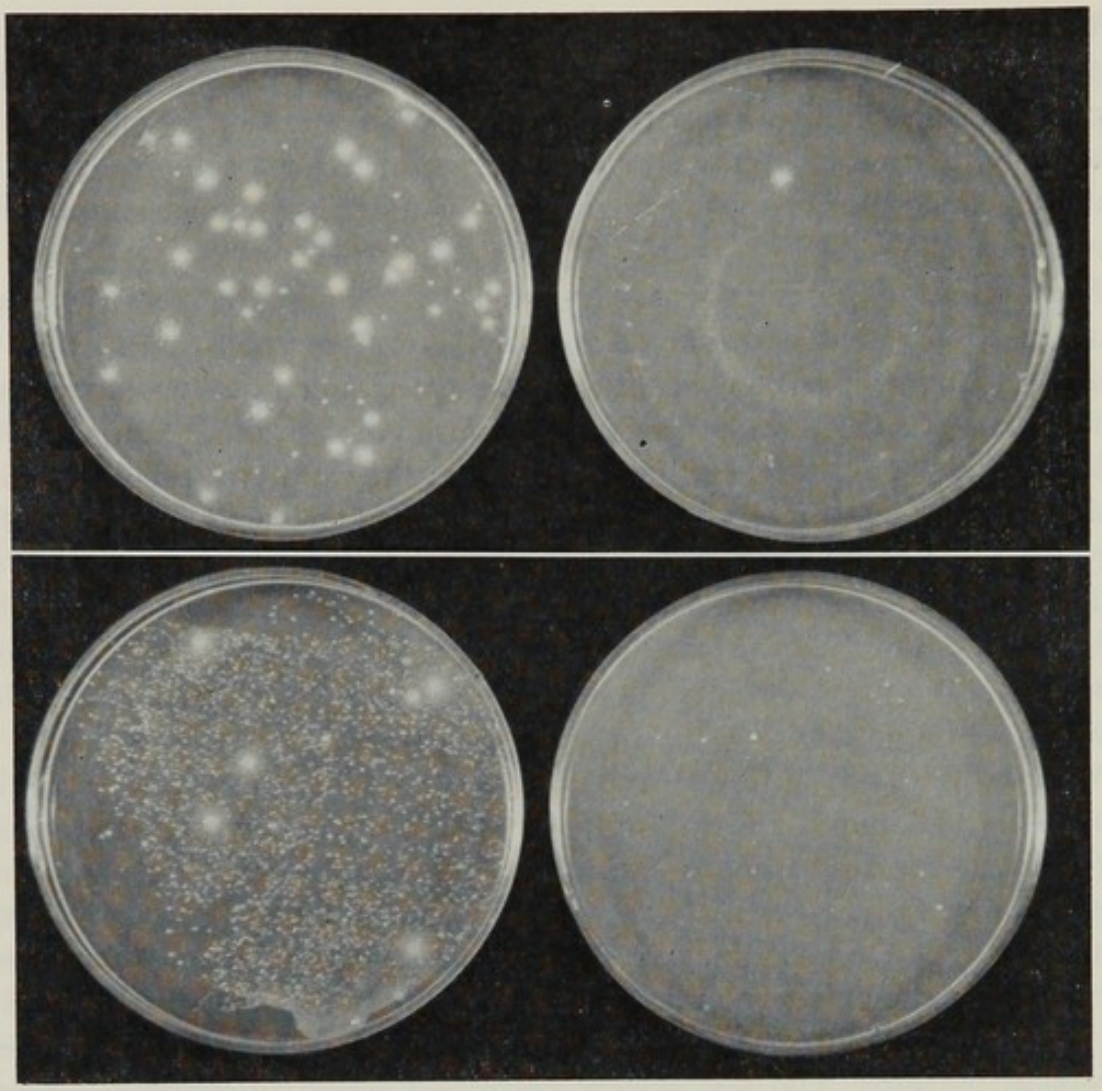

FIG. 4—Reversions of an ethyl methanesulfonate induced *lac− (i−z−)* mutant of *E. coli*. Top left—induced by 2-aminopurine; top right—spontaneous mutations; bottom left—induced by ethyl methanesulfonate, bottom right—ultraviolet induced. Photographs taken after 5 days incubation on medium containing filter-sterilized lactose as a carbon source (experiment by Mr. Glenn Hodges).

All 38 EMS induced strains gave mostly small colony revertants when treated with EMS (Fig. 4) whereas treatment with aminopurine induced mostly large colony revertants. These data (Table 6) are best interpreted by supposing that EMS induces mainly a G:C to A:T transition with only an occasional A:T to G:C change. Initial reaction of EMS with G to yield an A:T pair eventually would mean that only rarely would an EMS induced mutant be expected to revert upon treatment with EMS. EMS was unable to induce a high frequency of true back mutations of 17 ultraviolet induced mutations and ultraviolet treatment did not induce revertants of the EMS induced strains. Schwartz *(loc. cit.)* interprets these results as indicating the production of transversions (or some change other than transitions) by ultraviolet irradiation.

TABLE 6—*Mutagen Specificity in Bacteria*

(Summary of Reversion Patterns Obtained with Ultraviolet and Ethyl Methanesulfonate induced Mutants of *Escherichia coli* using ethyl methanesulfonate (EMS), diethyl sulfate (ES) ultraviolet irradiation (UV) and aminopurine (AP) to induce reversions)

| Primary Mutagen | Secondary Mutagen | Response | Revertant Colony Type |
|---|---|---|---|
| | UV | O/+ | small; nonfeeders |
| EMS (38 mutants) | EMS | ++++ | small; nonfeeders |
| | AP (12 tested) | ++ | large; feeders |
| | UV | +++ | large; small; feeders; nonfeeders |
| UV (17 mutants) | | | |
| | EMS + ES | ++++ | small; nonfeeders (some feeders) |
| | AP (4 tested) | + | small; nonfeeders (some feeders) |
| | UV | +++ | large; feeders |
| Spontaneous (1 mutant) | EMS | +++ | mostly large; feeders |
| | AP | + | small; nonfeeders |

Data from Schwartz, 1963. Feeders and nonfeeders refer to the type of lac+ reversions (Fig. 4). Small and large colonies refer to prototrophic reversions of auxotrophic mutants.

Most of the investigations on EMS specificity have employed the rII mutants of T4 bacteriophage. One of the findings (Green and Krieg, 1961) is that mutations induced by EMS are delayed; that is, they occur during the course of phage replication inside the host bacterium. This implies that alkylated DNA can replicate, a finding confirmed by recent studies on the replication of *B. subtilis* DNA after treatment of the cells with methyl methanesulfonate (Strauss, 1963). The finding also accounts for the observation that alkylation tends to produce mosaic type mutants in Drosophlia (Auerbach, 1951). If an alkylated strand participated in several rounds of replication, errors could occur at any DNA division and mosaics would result. Krieg (1963) has shown that EMS induces the reversion of many analog (and some EMS) induced mutants. Mutants induced to revert by ethyl ethanesulfonate and not by base analogs do occur rarely (Freese, 1961) and their reversion frequencies with ethyl ethanesulfonate are low compared to the spontaneous frequency.

Most of the ethylation induced mutations are therefore transitions and most of these represent G:C to A:T transitions. There may be a small

proportion of changes induced that are not transitions and some of these may represent the class of transversions. Operationally transversions represent the class of mutants which are *not* transitions. Small additions or deletions would act as transversions. This preponderance of transitions should not be expected if depurination is a step in the production of mutations since there are two chances of producing a mutagenic transversion for every chance of producing a mutagenic transition (one of the transition type insertions results in restoration of the original) unless there is some kind of restriction in base sequences by which neighboring bases guide the incorporation of purines or pyrimidines. It *does* seem that the nearest neighbor frequencies in DNA are non-random (Josse et al., 1961) and there are experiments which suggest that pyrimidine "runs" in DNA occur more frequently than expected (Shapiro and Chargoff, 1960; Burton and Peterson, 1960). However, the alternate hypothesis of mispairing due to tautomeric changes in an alkylated guanine residue suggests in a direct way that transitions should be the major type of mutagenic change produced and I prefer this hypothesis.

Two additional arguments support the mispairing hypothesis. Storage of DNA under conditions which promote depurination should increase the frequency of mutation; no such frequency increase has been obtained (Strauss, 1961). In addition it is unlikely that apurinic DNA can reproduce *in vivo* and therefore unlikely that it can serve as a template upon which errors could be made. The evidence for the lack of apurinic acid replication is indirect. Heating at temperatures below the thermal denaturation point inactivates alkylated DNA. Alkylated DNA does not appear to have single strand breaks after heating as compared to alkylated DNA which has been inactivated for transformation by treatment with an enzyme from *M. lysodeikticus* specific for partially denatured DNA (Strauss, 1962). Ultraviolet radiation makes transforming DNA susceptible to inactivation by this enzyme even though DNA treated with ultraviolet is not heat sensitive. It therefore seems reasonable to suppose that heating alkylated DNA below the thermal denaturation point inactivates DNA by a mechanism different from enzyme treatment. If the enzyme inactivates by causing breaks (Fig. 5) then most probably heating alkylated DNA inactivates by depurination. Alkylated DNA can replicate (Strauss, 1963) and the data show that DNA with either breaks or depurinations (not specifying which) does not replicate. If one makes the assumption that ability to transform implies ability to replicate and the reverse, it is evident that apurinic acid, unable to transform would be unable to replicate *in vivo* and therefore unlikely to participate in mutagenesis. We do not yet know how many apurinic sites must be produced before *in vivo* replication is inhibited.

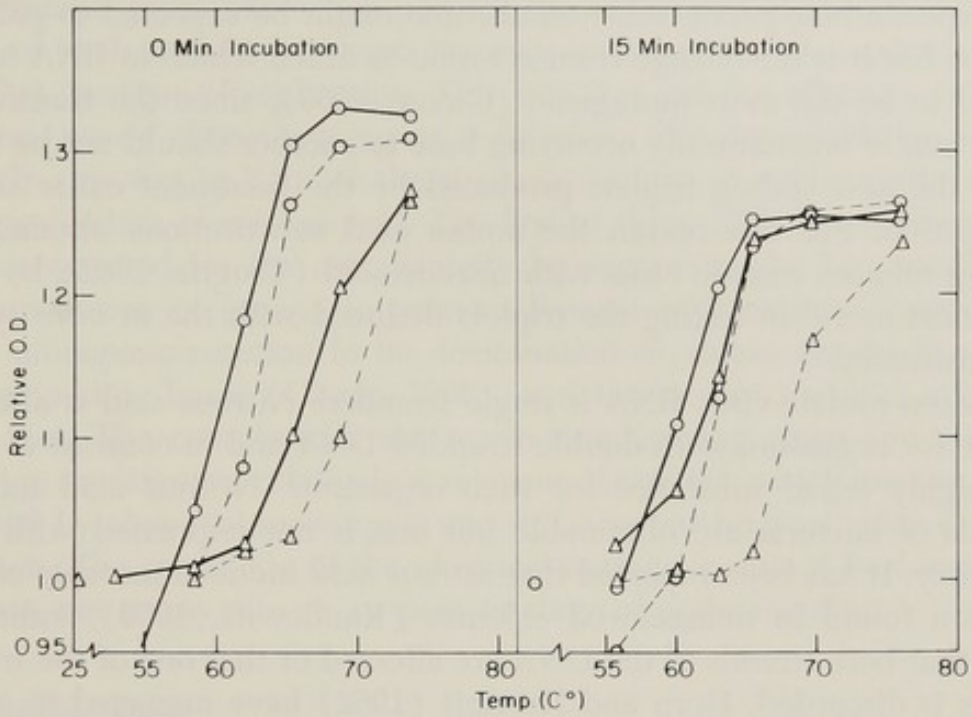


FIG. 5—Reversible and irreversible denaturation curves (Geiduschek, 1962) for *B. subtilis* DNA alkylated for 120 minutes with 0.05 M methyl methanesulfonate and treated with the degradative enzyme from *M. lysodeikticus* (Strauss, 1962) for 0 minutes (left hand) or 15 minutes (right hand). Solid lines—alkylated; broken—nonalkylated control. Circles—measured at ambient temperature, triangles—measured at room temperature after quenching in ice. "Collapse" of the curves after 15 minutes indicates a deoxyribonuclease type action. Buffer = $5 \times 10^{-3}$ M NaCl, $10^{-3}$ M $NaH_2PO_4$, $10^{-4}$ M ethylenediaminetetracetic acid, pH 7.0.

What is really evident from a consideration of the EMS data is that it is impossible to specify the change responsible for the mutation of a particular organism. It is possible to predict that a large proportion of EMS induced mutations will be G:C to A:T transitions but it is not possible to say that any *particular* mutant is a G:C to A:T transition without further tests on that strain.

### *B. Nitrous Acid*

Nitrous acid reacts with cytosine to produce uracil, with adenine to produce hypoxanthine and with guanine to produce xanthine. Since both cytosine and uracil are naturally occurring bases in RNA, nitrous acid might be expected to produce dramatic effects on organisms utilizing RNA as genetic material. It is generally considered that the guanine to xanthine transformation is lethal, that is, it produces nonviable nucleic acid.

The hypoxanthine produced from adenine might be expected to pair like guanine but it is the change from cytosine to uracil which in RNA is considered to be the most mutagenic (Gierer, 1960), since the transformation *in situ* of one naturally occurring base to another should not be lethal unless the new coding triplets produced by the treatment cause serious malfunction. For this reason the amino acid substitutions obtained by treating tobacco mosaic virus with nitrous acid (Tsugita, 1962a, b) have been most useful in testing the triplets deduced with the *in vitro* system for consistency.

Tobacco mosaic virus RNA is single stranded. Nitrous acid is also mutagenic for organisms with double stranded DNA and in contrast to EMS it is highly lethal substance for such organisms. Nitrous acid induced mutants of bacteria are obtainable but one is not impressed with their frequency. It has been reported that nitrous acid induced mutants of bacteria are found in nonsectored colonies (Kaudewitz, 1959), indicating either that both strands of the DNA are affected or that one of the treated strands is discarded. Horn and Herriott (1962) have managed to obtain mutations by treating transforming DNA with nitrous acid *in vitro* but in order to get such mutations in good yield it was necessary to denature the DNA, treat the single strands with mutagen and then recombine the two strands for transformation. While it does seem that the nitrous acid induced mutations of tobacco mosaic virus are to be accounted for as single base substitutions, it is also apparent that this need not be true for all bacteriophage mutants and, one would suspect, not necessarily true for any organism with a double stranded DNA (Barnett and deSerres, 1962; Case, 1962). Tessman (1962) has reported, for example, that about 8 per cent of the mutations produced in the *r* region of bacteriophage T4 by nitrous acid represented fairly large deletions. One cannot use the production of reversions after treatment of a mutant with nitrous acid alone as evidence for the transition character of a particular mutation.

The structural basis for the complexity in nitrous acid reactions with double stranded DNA has been somewhat clarified by the work of Geiduschek and his collaborators and by Luzzati (Geiduschek, 1961; Luzatti, 1962; Becker and Geiduschek, 1963; Zimmerman and Geiduschek, 1963). Apart from its deaminating action, nitrous acid produces covalent crosslinks between the two strands of DNA. These bonds (of as yet unknown detailed structure) can be demonstrated by subjecting nitrous acid treated DNA to denaturation and then showing that the strands specifically recombine. The demonstration can be made most elegantly by showing that the residual activity of transforming DNA after nitrous acid treatment is resistant to conditions of heating which denature native DNA. These findings imply that the DNA, after treatment with nitrous

acid contains crosslinks which prevent the separation of the strands. The result of such inability to separate may well be loss of a portion of a strand at the time of replication. This loss, if it did not affect an essential function, would then show up as a deletion. The demonstration is particularly amusing in light of the previous history of explanations of the biological action of nitrous acid. The first of these (Thom and Steinberg, 1939) accounted for the mutagenicity by supposing the key reaction to be deamination of groups in protein. Present explanations suppose the major mutagenic reaction to be deamination of amino groups in purine and pyrimidine bases (Gierer, 1960), and the newest material adds another fact. The complexities are worth emphasizing since any induced mutation represents a relatively rare event. Reactions which are not easily detected by chemical techniques can still account for particular cases of mutation. The specificity of the chemical mutagens cannot be considered absolute, not at any rate at our present stage of understanding.

### *C. Acridine Dyes*

Proflavin and similar acridine dyes are mutagenic for vegetative bacteriophage (Orgel and Brenner, 1961) but it has not been demonstrated that these substances produce mutations in bacteria or other organisms. The mutations produced in T4 after proflavin are similar to the majority of spontaneous mutations in that they are not induced to revert by treatment with bromouracil or aminopurine. It was this behavior that induced Freese (1959) to suppose that since proflavin induced mutations were obviously not transitions they must represent some different class of mutations; he supposed that they must therefore be what he called transversions. Freese supposed that proflavin spread the two chains far enough to permit a purine opposed to a purine to fit into the DNA structure. From physical studies of the reaction of DNA with acridines Lerman (1961, 1963) suggested that these mutagens act on DNA by "intercalation into the helix by extension of the backbone." The hypothesis of Brenner et al. (1961) was that insertion of the acridine dye resulted in either addition or deletion of one or a few base pairs at the time of replication.

There is no real evidence that proflavin or similar mutagens work by either one or the other mechanisms. What is known is that the hypothesis of addition or deletion accounts most elegantly for the suppressor action of certain proflavin and spontaneous mutations when they are combined with other mutants of similar type (see above and Crick et al., 1961). The main justification for this mechanism of mutagenesis is that it is suggested by the set of phenomena that it was designed to explain!

*D. Conclusion*

It is certainly possible to gain an idea of what has happened in a primary mutation by testing the reversion specificity of the mutant obtained (Table 6). Transition type changes can probably be identified with some certainty; it may perhaps be possible to distinguish G:C to A:T from A:T to G:C transitions. The class of transversion mutants is one for which the type of change cannot be specified by further mutation studies. If a change occurs which is not revertible at high frequency by treatment with a base analog or EMS and which results in the production of a mutant protein with some residual activity and if analysis of the amino acid sequence of the protein indicates substitution of a single amino acid which can be accounted for on the basis of results with the *in vitro* system, then the mutagen probably induced a transversion in Freese's original sense. Without the chemical analysis of the protein, or if no detectable (active) protein were produced it would, at present, be necessary to reserve judgement as to whether there was a transversion or an addition or deletion as suggested by Brenner et al. (1961). The problem will be settled shortly since numerous lysozyme deficient mutants of phage T4 induced both by proflavin and by other mutagens are available (Streisinger et al., 1961) and a sequence analysis of this small protein can be expected shortly.

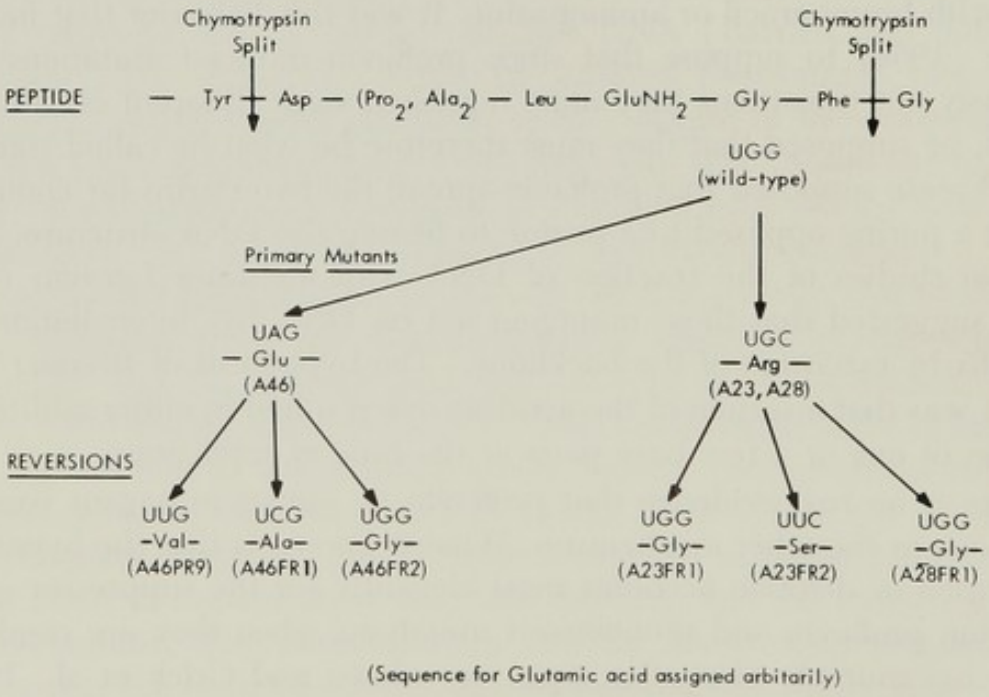


FIG. 6—Amino acid substitutions in the tryptophan synthetase A protein of *E. coli* (Henning and Yanofsky, 1962)

## VII. Amino Acid Replacements as a Method of Determining Base Order

There are now sufficient data to illustrate the method by which substitution data can be used to give an idea of base sequence and to test codes suggested by the *in vitro* system (Tsugita, 1962a, b). However, any test is based on some assumption about the nature of the code.

Bacterial mutants with altered tryptophan synthetase A protein have been isolated and studied by Yanofsky and his group (1961). Analysis of the protein "fingerprints" led to the recognition of a series of ultraviolet induced mutants in which a glycine residue was substituted to give two strains, one with a glutamic acid residue at the glycine position, the other with an arginine (Fig. 6). From the glutamic acid substituted mutant the following spontaneous revertants were obtained: a partial revertant with valine substituted for glutamic acid at the glycine position, and two (phenotypic) full revertants, one containing alanine and one the original glycine. From the arginine substituted mutant there were obtained two (phenotypic) full revertants, one with the original glycine and one with the amino acid serine inserted at the glycine position (Henning and Yanofsky, 1962). If one assumes a) the coding triplets proposed by Speyer et al. (1962), b) that the arginine and glutamic acid substitutions represent primary mutations at adjacent bases, and c) that the nucleotide sequence for glutamic acid is UAG, then one can write a unique nucleotide order for the triplets coding for glycine, arginine, valine, alanine and serine. The unique feature of the Henning and Yanofsky (1962) data is that it is necessary to assume that (at least) adjacent bases are involved in the arginine and glutamic acid primary mutations since a normal (glycine) containing recombinant was isolated by crossing the two primary mutants. Such a recombinant could not be obtained unless the mutations occurred at different base pairs. It might be noted that the suggested substitutions suppose that both ultraviolet induced and spontaneous mutants may be either transitions or transversions.

Continued collection of mutations and substitutions presumably will lead to an order of nucleotides for all the amino acids. An exercise in which base orders are determined on the basis of the Henning and Yanofsky (1962) data is shown for human hemoglobin in Figure 7. It is very unlikely that the suggested nucleotide orders have real meaning. The base sequences are based on coding triplets obtained before the technical problem of getting amino acid incorporation with polymers that did not contain U had been solved. Since triplets are being found (Bretscher and Grunberg-Manago, 1962; Gardner et al., 1962; Jones and Nirenberg, 1962) which do not contain U it may be possible to account for the substitutions

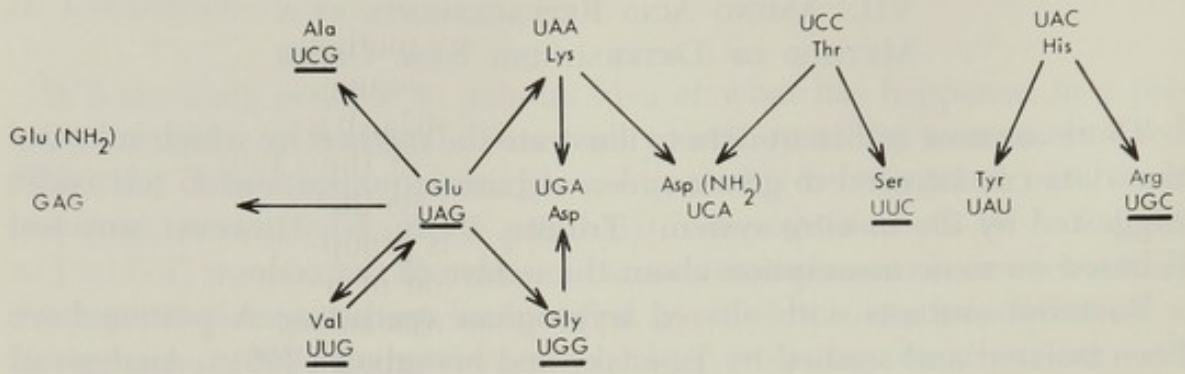


FIG. 7—An exercise: Determination of base sequence in coding triplets using the method suggested by Smith (1962) and (1) base sequences suggested by Henning and Yanofsky (1962); (2) amino acid substitutions in hemoglobin as listed by Smith (1962); (3) triplet code letters of Speyer et al. (1962) and of Gardner et al. (1962); and (4) Positioning of Crick (1963) (the underlined orders are those suggested by Henning and Yanofsky, 1962).

using different triplets, indeed the insertion of U into many of the triplets is based on the theoretical bias that U *must* be present. It probably will not be possible to make permanent assignments until the complete set of triplets—if triplets they be—is known (Hendler, 1962). Furthermore, it is likely that more extensive substitution data, coming particularly from known mutational steps, will be necessary. As Henning and Yanofsky (1962) point out, the substitutions found in nature, *i.e.*, in hemoglobin, may represent second step changes just as the glycine-serine substitution in tryptophan synthetase A protein (Fig. 6) has arginine as an intermediate.

If the base order is as suggested by Henning and Yanofsky (1962) and if recombination can be detected between the different mutants, then it should be possible to obtain a strain with an amino acid inserted at a position where neither of the parental strains have this particular amino acid sequence. For example, a cross of valine X arginine at position 8, represented as UUG X UGC should give a UUC recombinant which according to the scheme would have serine at position 8 and a UGG recombinant which would have glycine. Such recombinants have in fact been found as have others predicted by the theory. The distinction between mutation and recombination is therefore even harder to make (see discussion in Strauss, 1960).

It is evident that amino acid replacement data coupled with genetic analysis is now the most promising method of testing codes and of eliminating particular ideas. Each successive review adds more data and the suppositions become more specific. The replacement data are being analyzed, on the whole, in terms of the triplet code and this concept is

based, as yet, only on a theoretical bias. We are rapidly approaching the time at which the data will actually specify the nature of the code.

TABLE 7—*Specificity of Some Mutagenic Agents*

| Mutagen | Major Reaction (s) | Type of Change | Organisms |
|---|---|---|---|
| 5-Bromouracil | Thymine analog Mispairing | A:T ⇄ G:C | Bacteriophage<br>Bacteria *(Salmonella)* |
| 2-Aminopurine | Adenine analog Mispairing | A:T ⇄ G:C | Bacteriophage<br>Bacteria *(E. coli, Salmonella)* |
| $HNO_2$ | Deamination (C → U, G → X, A → HX) cross-linking | C → U<br>A:T ⇄ G:C<br>Deletion | RNA viruses<br>Bacteriophage<br>*E. coli*<br>*Neurospora* |
| $NH_2OH$ | Cytosine destruction | G:C ⇄ A:T | Bacteriophage |
| Ethyl methane-sulfonate | Formation of 7-ethylguanine | G:C ⇄ A:T | Bacteriophage<br>Bacteria *(E. coli, Salmonella, B. subtilis)*<br>*Neurospora*<br>Barley |
| Proflavin | Insertion in DNA molecule | Additions and deletions or transversions | Bacteriophage |

References: Freese (1959); Champe and Benzer (1962); Freese, E., Freese, E. B. and Bautz (1961); Lawley and Brookes (1961); Krieg (in press); Brenner et al. (1961); Gierer (1960).

Abbreviations:

A = adenine
C = cytosine
T = thymine
U = uracil
G = guanine
X = xanthine
HX = hypoxanthine

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Chapter 2

# Pharmacogenetics

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## Pharmacogenetics

Pharmacogenetics deals with drug responses and their modification by hereditary influences. The elucidation of genetic mechanisms involved in drug effects is included in this term (Motulsky, 1957; Kalow, 1962; Evans, 1963).

The biotransformation of drugs and their ultimate disposal requires multiple enzyme-mediated reactions. Since genic action controls the activity of each enzymatic step in such reactions, variations in drug response would often be expected to have a genetic basis. Multiple reactions are usually involved in biotransformation of a drug. A typical sequence might include the following steps: absorption→plasma binding→drug-cell interaction→breakdown→conjugation→excretion. Minor genetic variation at each enzymatic step involved in these reactions may be additive and together with environmental variation may give a normal or unimodal distribution curve when measurements of drug decay (such as blood level after a specified time of half-life of the drug) or other parameters relating to the drug's action are considered. A normal Gaussian distribution curve may thus reflect the interaction of several genes affecting different steps of drug metabolism. Wide variation noted in salicylate blood levels after a standard dose (Evans and Clarke, 1961) may reflect such a mechanism. When a population can be categorized into clearly distinct classes by some measurement and a multimodal distribution curve is obtained, a single major gene involved in drug metabolism may be sus-

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pected. If such classes have variable frequencies in different populations or racial groups, existence of a major gene affecting the drug's metabolism is suggested. However, a unimodal distribution does not rule out the operation of a single major gene if the measurement under study does not reflect the action of the mutant gene very directly. Even quantitative enzyme assays may not be sufficiently sensitive, and it may be necessary to devise tests indicative of the structural alteration of a mutant enzyme affecting drug metabolism (see Chapter 3).

Investigations of monozygotic and dizygotic twins may point to genetic factors in biotransformation of a drug (Bönicke and Lisboa, 1957; Sunahara, 1961), but will be of little help in elucidating the genetic mechanism involved.

## Isoniazid Metabolism

Metabolism of isoniazid (INH) is affected by a single major gene involved in INH acetylation. When blood levels of isoniazid after administration of a standard dose (such as 4 mg. per Kg.) were measured in a group of individuals, the distribution of blood levels after 6 hours followed a bimodal distribution (Mitchell, Riemensnider, Harsch and Bell, 1958; Knight, Selin and Harris, 1959). Individuals with high and low blood levels were classified as slow and rapid inactivators, respectively. These differences in phenotype are unrelated to intestinal absorption, protein binding, renal glomerular clearance, or renal tubular reabsorption (Jenne, MacDonald and Mendoza, 1961). Urinary excretion studies indicated that in rapid inactivators more of the drug could be recovered as the acetylated derivative (see Schmidt, 1961 for ref.). When liver slices of rapid and slow inactivators were incubated with acetate, isoniazid, CoA and ATP, isoniazid disappeared from the mixture at a faster rate from the specimen of rapid inactivators. These findings suggest that slow inactivators lack a hepatic acetylating enzyme (Evans, 1962). This postulated enzyme does not acetylate all drugs which undergo acetylation. Red blood cell suspensions supplied with acetate, CoA, and ATP acetylate PABA and PAS but never INH (Motulsky and Steinmann, 1962). There was no evidence of polymorphism when PAS acetylation by red cells was studied since a unimodal distribution curve was found (Fig. 1). The lack of INH acetylation and the presence of PABA and PAS acetylation in the red cell suggest that different acetylating enzymes are involved. Other data support this concept. The disappearance rate of PAS in the plasma bears no relation to INH inactivation status (Jenne et al., 1961), and the urinary excretion of PAS shows a unimodal rather than a

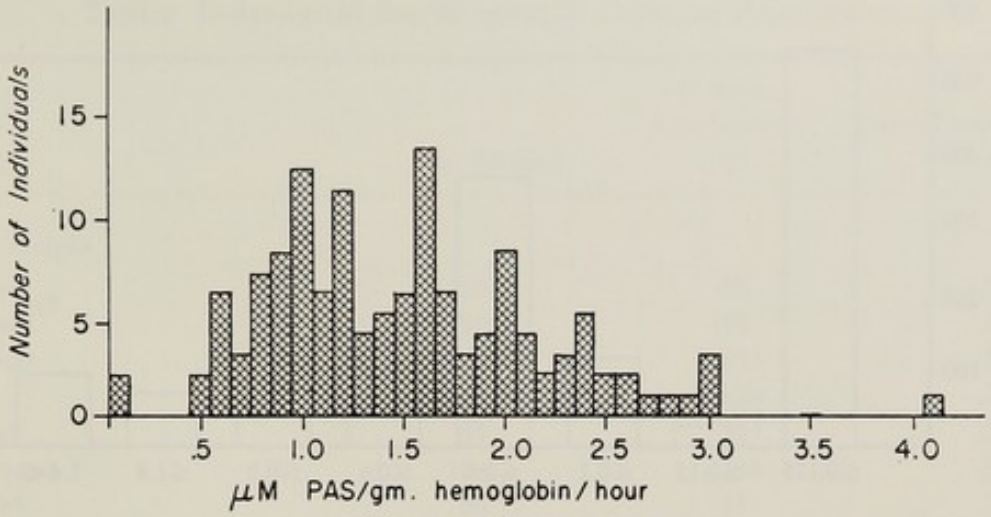


FIG. 1—Acetylation of PAS by human hemolysates. Reaction mixture contained .50 ml. CoA (1μM); .50 ml. ATP (5μM); .25 ml. 1 M Tris pH 7.99; .15 ml. 0.1 M potassium citrates; .30 ml. 2.43 mM PAS (.73μM); 1.00 ml. hemolysate (12 Gm. Hb / 100 ml.).

bimodal distribution (Evans, 1963). On the other hand, when urinary excretion of sulfadimidine was measured, rapid INH inactivators excreted less of the free drug than slow inactivators and the distribution of drug excretion was bimodal, suggesting that the INH-acetylating enzyme is involved in biotransformation of this sulfa drug (Evans, 1962). Sulfisoxazole presumably also is acetylated by the polymorphic enzyme (Sunahara, 1961).

Family studies by several groups of investigators indicate that slow inactivators are homozygous and presumably lack the "INH-acetylating" enzyme (Knight et al., 1959; Evans, Manley and McKusick, 1960; Sunahara, 1961; Sunahara et al., 1961; Motulsky, unpublished). The offspring of two slow inactivators are always slow inactivators. Carriers of a single or double dose of the gene for the acetylating enzyme cannot be differentiated by most methods of INH assay and have been classified as rapid inactivators. With recent refinements of microbiologic assay for INH, a clear trimodal curve of isoniazid blood levels has been obtained, thus differentiating all three genotypic classes (Sunahara, 1961; Sunahara et al., 1961) (Fig. 2).

The gene frequency of "INH acetylase" deficiency (slow inactivator type) has been determined in many populations (Armstrong and Peart, 1960; Harris, 1961; Sunahara, 1961; Sunahara et al., 1961; Evans, 1962; Mitchell et al., 1960; Devadatta et al., 1960; Gangadharam and Selkon, 1961; Schmiedel, 1961; Szeinberg et al., 1963). Table 1 lists homozygote frequency and gene frequency of the enzyme deficiency. The gene has the lowest frequency in Eskimos, is less widespread in Far Eastern popu-

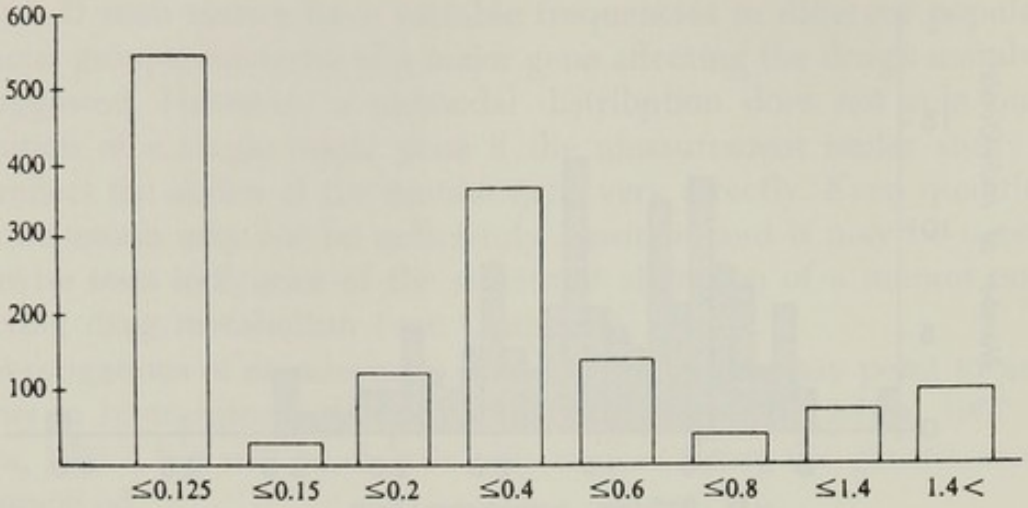


FIG. 2—Frequency distribution curve of 6 hour blood levels of INH (microbiologic method of Sunahara) after single dose of 4 mg. Kg. INH in 1,386 Japanese over 20 years of age (from Sunahara 1961). Note bimodal distribution and relative paucity of "slow inactivators" in this Japanese population.

lations such as in Japanese and Chinese, but is quite common in Negroes and various European populations, where two-thirds to four-fifths of the population carry the deficiency gene in either homozygous or heterozygous state.

The therapeutic significance of the INH polymorphism relates to toxic polyneuropathy induced by INH therapy. Such a complication is practically limited to slow inactivator homozygotes, i.e., individuals with high blood levels of the drug (Hughes, Biehl, Jones and Schmidt, 1954). Although it has been suggested that tuberculosis in rapid inactivators might respond less favorably to INH therapy, extensive clinical studies have failed to demonstrate such a relationship (see Evans, 1963).

## Suxamethonium Sensitivity

Suxamethonium is a muscular relaxant widely used by anesthesiologists and by psychiatrists. Prolonged apnea following suxamethonium may be caused by heredity pseudocholinesterase deficiency. This topic is discussed in detail in Chapter 3.

We have studied two additional patients with complete absence of pseudocholinesterase activity and presumably homozygous for the "silent" gene (Hodgkin, Giblett, Levine and Motulsky, 1963). Another such patient has also been detected by Austrian investigators. A liver biopsy in this individual showed no pseudocholinesterase activity (Doenicke, Gurtner, Kreutzberg, Remes, Spiess and Steinbereithner, 1963). On starch gel electrophoresis of serums stained for cholinesterase, individuals with

TABLE 1—*Isoniazid Inactivation in Different Populations*

| | No. Studied | % Slow Inactivators $q^2$ | Gene Frequency q |
|---|---|---|---|
| *Asiatic Origin* | | | |
| Eskimos[1] | 226 | .05 | .22 |
| Japanese[2] | 30 | .10 | .32 |
| Japanese[3] | 1808 | .115 | .34 |
| Ainu[3] | 86 | .128 | .36 |
| Korean[3] | 65 | .108 | .33 |
| Ryukuyan[3] | 124 | .145 | .38 |
| Chinese[4] | 85 | .15 | .39 |
| Thais[3] | 108 | .278 | .53 |
| American Indians[5] | 15 | .21 | .46 |
| Hindu Indians[6] | 143 | .58 | .76 |
| Hindu Indians[7] | 299 | .60 | .77 |
| *African Origin* | | | |
| American Negro[2] | 95 | .42 | .65 |
| American Negro[5] | 31 | .48 | .69 |
| Sudanese Negro[4] | 102 | .65 | .80 |
| *European Origin* | | | |
| U.S. Whites[2] | 112 | .47 | .69 |
| German[8] | 524 | .44 | .66 |
| U.S. Scandinavian[2] | 70 | .67 | .82 |
| U.S. Italians[2] | 14 | .64 | .80 |
| U.S. Greek[2] | 10 | .60 | .77 |
| "Spanish Americans"[2] (Salt Lake City)* | 23 | .60 | .77 |
| "Spanish Americans"[5] (Denver)* | 131 | .30 | .55 |
| *Jewish Groups* | | | |
| U.S. Askenazi[2] | 11 | .55 | .75 |
| Israeli Askenazi[9] | 100 | .67 | .82 |
| Israeli Non-Askenazi[9] | 179 | .69 | .83 |
| Israeli Baghdad Jews[9] | 60 | .75 | .87 |

(1) Armstrong & Peart, 1960.
(2) Harris, 1961.
(3) Sunahara, 1961.
(4) Evans, 1962.
(5) Mitchell et al., 1960.
(6) Deadatta et al., 1960.
(7) Gangadharam & Selkon, 1961.
(8) Schmiedel, 1961.
(9) Szeinberg et al., 1963.

* Variable degrees of Indian admixture in these "Spanish-American" populations probably explain the differences in gene frequency.

TABLE 2—*Mutants at Cholinesterase (E) Locus*°

| Genotype | | Phenotype | | Type of | Esterase Level | Typical Dibucaine | Typical Fluoride | |
|---|---|---|---|---|---|---|---|---|
| New Nomenclature† | Lehmann's Nomenclature° | New Nomenclature | Previous Designation | Enzyme Present | Rel % | No. | No. | Approximate Frequency |
| $E_1^u$ $E_1^u$ | N-N | U | "Usual" | u(sual) | 100 | 80 | 64 | 96% |
| $E_1^u$ $E_1^a$ | N-D | I | "Intermediate" | u + a(typical) | 78 | 62 | 48 | 4% |
| $E_1^a$ $E_1^a$ | D-D | A | "atypical" | a | 25 | 20 | 23 | 1/3000 |
| $E_1^s$ $E_1^u$ | S-N | U | usual | u | 65 | 80 | 64 | 1/150 |
| $E_1^s$ $E_1^s$ | S-S | S | "silent"; "zero" | none | 0 | — | — | 1/100,000 |
| $E_1^s$ $E_1^a$ | S-D | A | atypical | a | 20 | 20 | 23 | 1/8000 |
| $E_1^f$ $E_1^u$ | F-N | UF | $U_1$ | f(luoride-resistant) + u | 80 | 76 | 52 | ? |
| $E_1^f$ $E_1^f$ | F-F | F | | f | 50 | 67 | 34 | Very rare |
| $E_1^f$ $E_1^a$ | F-D | IF | $I_1$ | f + a | 60 | 50 | 30 | ? |
| $E_1^f$ $E_1^s$ | F-S | F | | f | Not described yet | | | ? |
| $E_2+E_2-$ | | $C_5+$ | | u + $C_5$ + | 130 | 80 | 64 | 5% |

° See also Table 4, Chapter 3.

† New nomenclature by agreement of workers in the field ($E_1$ = first allele at cholinesterase (E) locus; $E_1^u$ = usual enzyme; $E_1^a$ = *a*typical i.e. "dibucaine resistant" allele; $E_1^s$ = *s*ilent allele; $E_1^f$ = "fluoride-resistant" allele; $E_2+$ = non-allelic cholinesterase locus ($E_2$) determining additional cholinesterase isoenzyme ($C_5+$) (Harris et al., 1963).

no cholinesterase activity were found to lack the normally found 5 isozyme bands, suggesting that a subunit common to the various zones was affected by the mutation (Hodgkin et al., 1963). A gene-controlled enzymatic inhibitor could be ruled out by the fact that no excess inhibition of enzyme activity was found when normal sera were mixed with sera from patients lacking pseudocholinesterase activity. Immunodiffusion and immunoelectrophoretic studies indicated the lack of antigenically cross-reacting material in homozygotes for the silent gene. In contrast homozygotes for the dibucaine-resistant allele exhibited cross-reacting material. This finding is consistent with physicochemical, kinetic and inhibition studies suggesting that the mutation causing dibucaine resistance affects enzyme *structure*. Absence of cross-reacting material in homozygotes for complete cholinesterase deficiency probably indicates lack of even enzymatically inactive gene products. The mutation may affect controlling elements of the gene and cause complete failure of protein production. However, structural mutations resulting in alteration of both the enzymatically active and the antigenic sites cannot be ruled out on the basis of absence of cross-reacting material. Another possibility is that a structural mutation leads to alteration in tertiary structure of the protein which would cover up the antigenic site. Lastly, a structural mutation might alter a subunit of the enzyme in such a manner that normal association of subunits to give reactive enzyme might be impossible. Heterozygotes for the silent gene have slightly depressed serum cholinesterase activity (68 per cent of normal) with wide overlap with normal (Hodgkin et al., 1963; Harris, 1963).

The mutation which suppresses cholinesterase activity completely is most likely an allele of the other mutants at the cholinesterase locus (Harris, 1963). The frequency of heterozygotes for the silent gene among parents of suxamethonium-sensitive individuals makes it unlikely that this mutation is inherited at an independent locus. Under that hypothesis, considerably more homozygotes for the silent gene should be found among suxamethonium-sensitive individuals. The great rarity of this genotype [none was found among 30 suxamethonium-sensitive individuals in Kalow's material (Simpson and Kalow, 1963)] is more consistent with allelism. Knowing that 25 per cent of suxamethonium-sensitive patients are heterozygotes for both the atypical ($E_1^a$) as well as the silent ($E_1^s$) allele, the gene frequency of the silent gene can be calculated as 0.003.*

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* Ratio of $\frac{\text{Homozygotes for atypical gene}}{\text{heterozygotes for both atypical and silent gene}} = \frac{3}{1}$

If the frequency of the atypical allele is q (0.02 by population study) and the frequency of the silent allele is r, $\frac{q^2}{2qr} = \frac{3}{1}$ ; $r = \frac{q}{6} = 0.0033$

Heterozygotes for the silent gene allele would be found in approximately 1:150. Frequency of silent gene homozygotes would be 1:100,000, an estimate consistent with the rarity of this genotype. Heterozygotes for both the atypical and silent allele would be found in 1:8000 as compared with a frequency of the usual atypical homozygotes of 1:2500. Table 2 characterizes the various cholinesterase mutants and indicates the nomenclature used in this field.

## Glucose-6-Phosphate Dehydrogenase (G-6-Pd) Deficiency*

Glucose-6-phosphate dehydrogenase deficiency owes its discovery to the occurrence of severe hemolytic reactions in some Negro individuals on the administration of the antimalarial primaquine. A series of investigations showed that red cells from drug-sensitive individuals had deficient G-6-Pd activity. G-6-Pd is the first enzyme active in the oxidative pathway of carbohydrate metabolism and is required for the maintenance of reduced glutathione (GSH) in red cells. Many drugs, such as sulfa drugs and nitrofuran derivatives, cause premature red cell destruction in enzyme-deficient individuals. The mechanism of drug hemolysis probably involves depletion of glutathione from the red cells. The enzyme deficiency also predisposes to blood destruction following ingestion of fava beans. Hemolysis during infections, certain types of chronic nonspherocytic hemolytic anemia, and many cases of neonatal jaundice not explained by blood group incompatibility can also be related to G-6-Pd deficiency.

Family studies indicate that G-6-Pd deficiency is sex-linked. Detection is simple in males, where the normal and enzyme-deficient genotypes can easily be discriminated. A dye test devised in our laboratories several years ago (Motulsky and Campbell-Kraut, 1960) has proved reliable for the detection of enzyme deficiency in males. Many other tests are available (see Motulsky, 1964a). In females, three genotypes exist: normal and abnormal homozygotes as well as heterozygotes. Female heterozygotes usually have intermediate enzyme levels, but not all heterozygotes can be detected with even the best methods, since there is overlap with normal enzyme levels. The sex-linked inheritance explains the greater frequency of G-6-Pd associated hemolytic disease in males.

Enzymatic and electrophoretic studies demonstrate six mutants of G-6-Pd deficiency (Kirkman, 1963). Four of these mutants are rare, including two variants associated with chronic nonspherocytic anemia (type Chicago I and Oklahoma I). The common mutations are the moderate

* See Beutler, 1959; Tarlov et al., 1962; Motulsky, 1964a for ref.

G-6-Pd deficiency in Africans and their U.S. descendants and the more severe deficiency in Caucasians, such as Greeks, Sardinians, or Sephardic Jews. In our studies, Oriental individuals such as Chinese and Indians had the more severe enzyme type of deficiency, but complete kinetic and electrophoretic studies have not yet been performed. Characterization of the various mutants is shown in Table 3.

TABLE 3—*Genetic Variants of G-6-Pd Dehydrogenase*

| | Enzyme Activity % | Electrophoretic Mobility | Lability | pH Optimum Curve | Substrate Requirements (TPN; G6P) |
|---|---|---|---|---|---|
| G-6-Pd def. (Africans) | 8-15 | Fast | N | N | N |
| G-6-Pd def. (Caucasians) | 3-6 | N | Sl.↑ | Wide | Sl.↓ |
| G-6-Pd def. (Oklahoma I) | 3-9 | ? | ↑↑↑ | Narrow | ↑↑↑ |
| G-6-Pd def. (Chicago I) | 3-9 | N | ↑↑↑ | N | N |
| G-6-Pd def. (Barbieri) | 40-60 | Very Fast | N | N | ↑ |
| G-6-Pd def. (Seattle I) | 8-15 | Slow | N | N | Sl.↓ |

N = normal; Sl. = slightly.

There is a general relationship between enzyme activity and the severity of clinical manifestations. Enzyme-deficient Caucasians presumably develop hemolytic anemia with lower dosage of drugs than Negroes with more moderate enzyme deficiency, and many more drugs have been implicated as hemolytic agents in the Caucasion groups. Similarly, favism never has been documented among Negroes.

*In vitro* measurement of enzyme activity alone may not give a full appreciation of the *in vivo* capability of the enzyme (Kirkman, 1963). Thus, the Oklahoma I mutant and the mutant common in Caucasians give similar degrees of enzyme deficiency, but the increased lability and substrate requirements of the Oklahoma mutant lead to fairly severe chronic blood destruction and anemia in the absence of external agents, while only mild shortening of red cell survival without anemia is seen in individuals with the Caucasian type of enzyme deficiency (Siniscalco, 1963).

All Negro enzyme-deficient subjects have a characteristic rapid electrophoretic phenotype (A) after staining for G-6-Pd activity following

starch gel electrophoresis (Boyer, Porter and Weilbacher, 1962). However, the identical electrophoretic phenotype (A) is also seen in 20 per cent of normal Negroes. The most likely interpretation is that this rapid electrophoretic phenotype reflects two different mutations, one causing G-6-Pd deficiency and the other sparing G-6-Pd activity.

Linkage studies in both Negro and Caucasian type of G-6-Pd deficiency have indicated close linkage (recombination fraction of 5 per cent) with the deutan type of colorblindness. These data strongly suggest allelism for the Negro and Caucasian type of G-6-Pd deficiency. The gene for the $X_g$A blood group is located further from the gene determining G-6-Pd deficiency (recombination fraction of 27 per cent) as shown by studies in both Negro and Greek enzyme-deficient subjects (for ref. see Motulsky, 1964a).

An X-linked enzyme deficiency provides an excellent tool for study of dosage compensation in X chromosomes. There are no significant differences between enzyme activity of normal males and females. When individuals with sex chromosomal polysomy such as XXX, XXY, XXXY are assayed for G-6-Pd activity, normal rather than increased activity is found. These findings are consistent with functional activity of only one X chromosome as suggested by the Lyon hypothesis. Indirect studies have suggested the presence of two red cell populations in heterozygote females with G-6-Pd deficiency as required by this hypothesis (Beutler, Yeh and Fairbanks, 1962). Tissue culture studies have confirmed these indications. Two distinct types of cells can be grown in clones from heterozygote females: (a) cells with enzyme deficiency and rapid electrophoretic phenotype and (b) cells with normal enzyme activity and normal electrophoretic phenotype (Davidson et al., 1963).

G-6-Pd deficiency occurs in many tropical and subtropical populations in gene frequencies up to 35 per cent. The geographic distribution of this sex-linked enzyme deficiency is very similar to that of the autosomal sickle cell trait and thalassemia. In a given population there is excellent correlation between the gene frequencies of G-6-Pd on the one hand, and sickling or thalassemia on the other (Allison, et al. 1963; Motulsky, 1960; Allison, 1961) (Fig. 3 and 4). Since different individuals are affected by these traits, half or more of a given population in certain areas of Greece or Africa may be carriers for one or the other red cell abnormality. The geographical distribution of enzyme deficiency and considerations of the growth requirements of the plasmodial parasite suggested that this trait was maintained in such high frequency by a selective advantage vis-à-vis falciparum malaria (Motulsky, 1960). Excellent correlation between previous malarial endemicity and gene frequency for

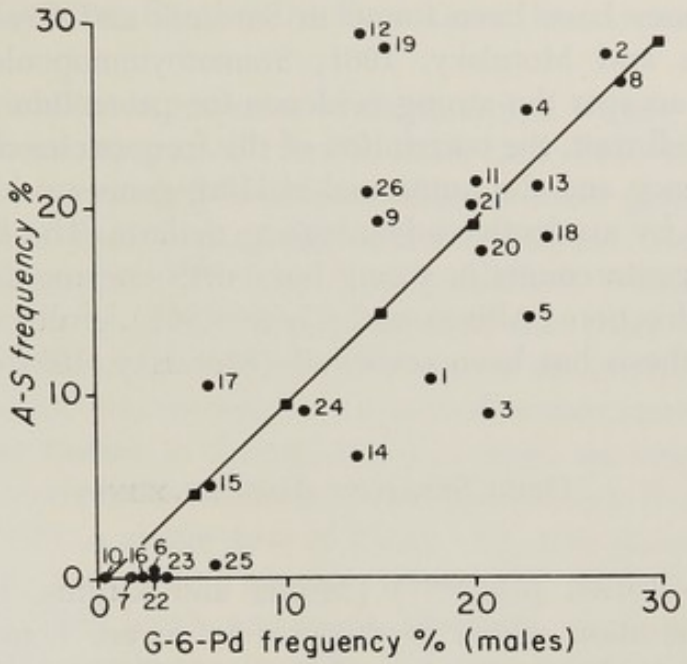


FIG. 3—Frequencies of G-6-Pd deficiency and sickling trait in various populations. Each numbered point represents one population (from Motulsky 1964a). The line represents the regression for these data.

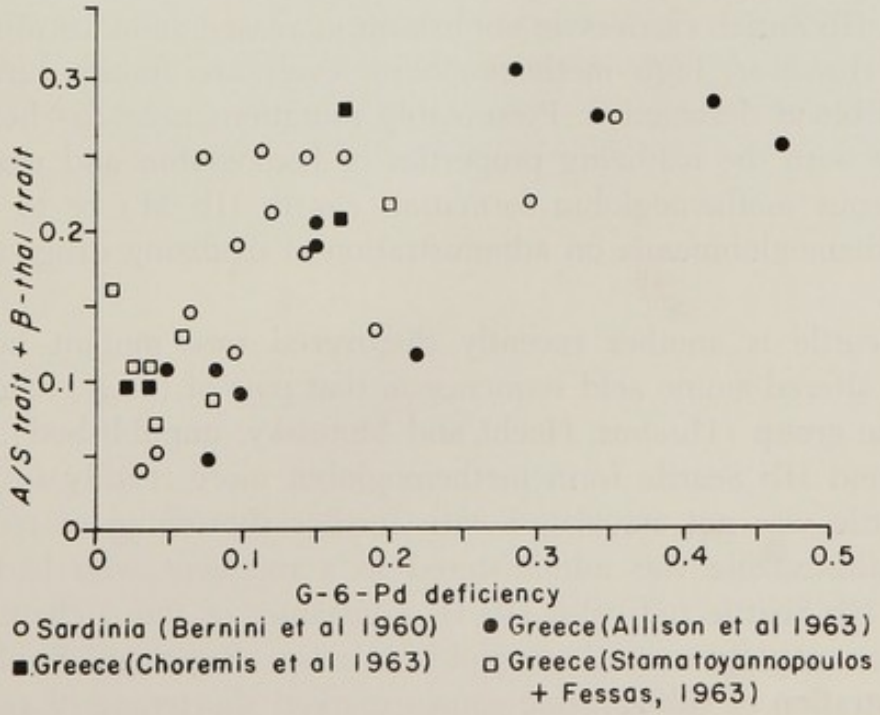


FIG. 4—Frequency of G-6-Pd deficiency (males) and beta-thalassemia trait in various villages in Sardinia and Greece. (Greek data give combined phenotype frequencies for sickling trait and beta-thalassemia.)

G-6-Pd deficiency have been found in Sardinia and Greece (Siniscalco, Bernini, Latte and Motulsky, 1961; Stamatoyannopoulos and Fessas, 1963). If one accepts the strong evidence for protection against malaria by the sickle cell trait, the correlation of the frequencies of the sex-linked G-6-Pd deficiency and the autosomal sickling gene would be difficult to explain except by a selective advantage to malaria. The finding of lower plasmodial parasite counts in young boys with enzyme deficiency points in the same direction (Allison and Clyde, 1961). Full evidence for the malarial hypothesis has been reviewed (Motulsky, 1964b).

## Drug Sensitive Hemoglobins

Hemoglobin Zurich ($a_2\beta_2^{63Arg}$) (Muller and Kirgma, 1961), is a rare hemoglobin mutation which predisposes to severe hemolytic reactions from sulfa drugs (Frick, Hitzig and Betke, 1962; Bachmann and Marti, 1962). The amino acid substituted by the Hb Zurich mutation is a histidine residue in the 63rd position on the $\beta$ chain. The heme group is attached to the $\beta$ chain at this position. Another mutant at the same site as well as other mutations in this general area of the hemoglobin molecule have caused various types of Hb M—the hemoglobin leading to hereditary methemoglobinemia (see Baglioni, 1963 for ref.). Unlike Hb M heterozygotes, Hb Zurich carriers do not exhibit increased amounts of methemoglobin. However, high methemoglobin levels are found during drug-induced blood destruction. Presumably mutations near the heme group interfere with the oxidizing properties of hemoglobin and may lead to spontaneous methemoglobin formation (as in Hb M) or to hemolysis and methemoglobinemia on administration of oxidizing drugs (as in Hb Zurich).

Hb Seattle is another recently discovered rare mutant hemoglobin with an altered amino acid sequence in that part of the $\beta$ chain opposite the heme group (Huehns, Hecht and Motulsky, unpublished). Both Hb Zurich and Hb Seattle form methemoglobin more readily *in vitro,* but Hb Seattle was not associated with further shortening of red cell life when sulfisoxazole was administered to a recipient who had received labeled Hb Seattle red cells. Hb H, a tetramer of the $\beta$ chain, also oxidizes more rapidly to methemoglobin *in vitro* (Rigas and Kohler, 1961). Administration of sulfisoxazole causes marked shortening of red cell life *in vivo.* Other drugs, such as sodium nitrite, were shown to precipitate Hb H *in vitro* and probably would produce more severe hemolysis *in vivo* (Rigas and Kohler, 1961).

## Genetic Factors in Dicumarol Metabolism

Marked variability in response to dicumarol, a widely used anticoagulant has long been recognized (Smith, 1938; Link, 1943-44). Early studies in rabbits were interpreted to indicate that recessive genes play a role in the biotransformation of dicumarol (Link, 1943-44). However, the full genetic data appear never to have been published. Weiner et al., (1950) showed in a study of several human subjects that there is marked variation in the rate of dicumarol decay. The rate of dicumarol metabolism for a given individual was constant on repeated administration. To test for possible genetic factors in dicumarol metabolism, we devised the following system of study (Gerstenberger, Campbell-Kraut, Fraser and Motulsky, unpublished). A single dose of 2 mg. / Kg. was given to the subjects and their family members. This dose is not high enough to significantly depress prothrombin levels and cause bleeding, yet is sufficient to give blood dicumarol levels measurable by a chemical method (Axelrod, Cooper and Brodie, 1949). Considerable variation in absorption, as previously shown with therapeutic doses of the drug (i.e., 5 mg. / Kg.), could be confirmed with the smaller doses. The slope of decay was determined from serial blood levels. The disappearance of the drug from the plasma followed an exponential rate after 24 hours. There was considerable variation in drug half-life with a mean T ½ of 25.2 ± 11 hrs ($\sigma$). No relation between age or sex of the subjects and their drug half-life could be demonstrated. However, there was a positive correlation between 24-hour value and drug half-life. This relation probably consists of two factors: (a) Individuals with longer half-lives will tend to have higher 24-

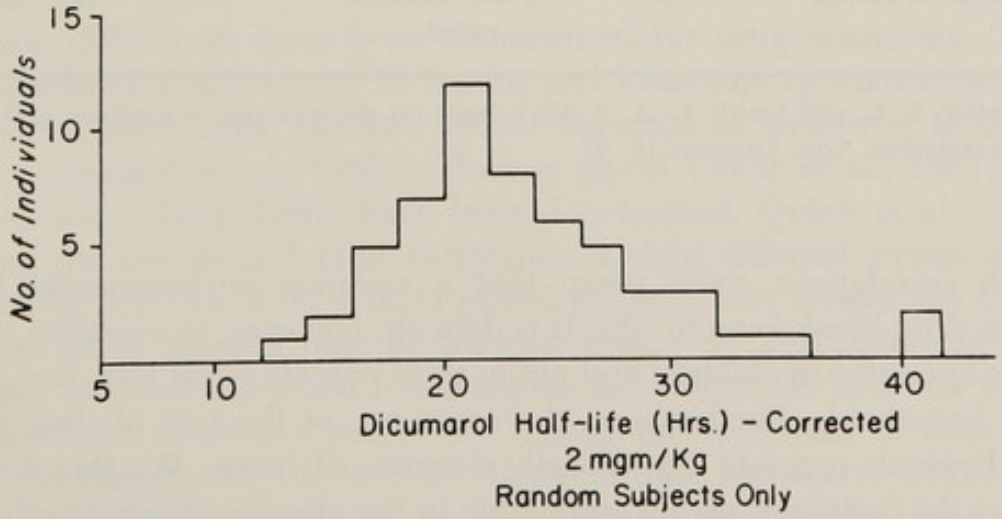


Fig. 5—Dicumarol half-life (corrected; see text) in hours in random subjects after 2 mg. Kg. orally.

hour values than those with more rapid drug destruction; (b) Weiner and his co-workers (1950) previously demonstrated that in a given individual a higher dose of the drug retards the biotransformation of dicumarol and gives a more prolonged half-life, suggesting that the biochemical system involved in drug metabolism could be saturated. The higher half-life found by other workers (O'Reilly, Aggeler and Gibbs, 1962) with higher doses of dicumarol also fit these earlier data. We argued, therefore, that individuals who absorbed more of the drug—as measured by the 24-hour blood value—would have a higher blood level and a longer half-life than those whose 24-hour value was lower. Consequently, we corrected the initially obtained T ½ for the 24-hour value. Considerable variation in half-life remained (mean T ½ : 23.6 ± 5.8 hrs) (Fig. 5). Attempts to fit the family data to a single-gene mechanism failed. In an attempt to assess the possible role of multiple genetic factors operative in this system, correlation coefficients (r) (Fisher, 1954) between relatives were computed. The sib-sib correlation of corrected dicumarol T ½ was 0.347 ± 0.091. No parent-child or midparent—midoffspring correlations could be demonstrated (Table 4). The finding of

TABLE 4—*Dicumarol Half-Life (Corrected)*

| Comparison | Pairs | Measure | r | Std. Error |
|---|---|---|---|---|
| Sib-Sib | 114 | Intraclass correl. (Weighted)* | + .347 | ± 0.091 |
| Parent-Child | 94 | Prod. moment correl. | — .077 | ± 0.102 |
| Midparent-Midchild | 20 | Prod. moment correl. | — .051 | ± 0.223 |

* Fieller, E. C. and Smith, C. A. B. 1951. Note on the analysis of variance and intraclass correlation. Ann. Eugenics 16: 97.

sib-sib correlations could mean that a common environmental factor affects drug breakdown in sibs. It is difficult, however, to visualize such a factor operative in children and not in their parents when they live in the same households. Furthermore, the constancy of the rate of drug decay in individuals suggests a genetically determined factor. We therefore interpret the finding of sib-sib correlation in the absence of parent-offspring correlation to indicate the operation of recessive genes in the breakdown of the drug. Their biochemical site of action is unknown.

## Species and Strain Differences

### *Drug Metabolism*

There are considerable species differences in biotransformation of drugs, and the fate of a drug in man cannot necessarily be predicted from animal experiments. Table 5 indicates the biological half-lives of several

Table 5—*Biologic Half-Life of Selected Drugs*

| Species | Hexobarbitone min. | Antipyrine min. | Aniline min. | Phenylbutazone hrs. |
|---|---|---|---|---|
| Man | 360 | 600 | — | 72 |
| Dog | 260 ± 20 | 107 ± 20 | 167 ± 66 | 6 |
| Rabbit | 60 ± 11 | 63 ± 10 | 35 ± 22 | 3 |
| Rat | 140 ± 54 | 141 ± 44 | 71 ± 1 | 6 |
| Guinea pig | — | 110 ± 27 | 45 ± 8 | 5 |
| Mouse | 19 ± 7 | 11 ± 0.25 | 35 ± 4 | — |

drugs in various species, including man (from Burns, 1962; Quinn, Axelrod and Brodie, 1958). Even if the rates of metabolism of a drug are similar in two species, different mechanisms of biotransformation may be operative. In the rabbit, ethyl biscoumacetate (Tromexan) is metabolized by hydrolysis of the ester group, while man inactivates the drug by hydroxylation. The biologic half-life is similar in the two species (Burns, Weiner, Simson and Brodie, 1953), although there is considerable variation in man.

Strain differences have been documented for drug sensitivity. There are considerabe differences in insulin and histamine resistance between strains of inbred mice (Brown, 1962). Variation in sensitivity to chloroform (Deringer, et al., 1953), ethylene glycol (Meir et al., 1963), and fluorouracil (Dagg, 1960) have been documented. Quinn et al. (1958) studied the metabolic fate of antipyrine in eight different strains of rats and found that two of these metabolized the drug very slowly. Jay (1955) studied mean sleeping time in different strains of mice following hexobarbitol administration and found significant differences between strains.

Differences in alcohol preference among inbred strains of mice have been demonstrated (for ref. see Rodgers and McClearn, 1962). The strain which had the highest alcohol preferences also had the highest liver alcohol dehydrogenase level—the key enzyme involved in the breakdown of alcohol.

*Teratogenic Agents*

Thalidomide causes limb malformations and other congenital malformations in man when taken during early pregnancy. In the rat, the drug causes fetal resorption and dead fetuses but no malformations or only minor ones (see Felisati, 1962, for ref.). In rabbits and mice, malformations are found with fairly high doses of thalidomide, although in rabbits a 75-fold increase in dose of thalidomide only causes a 3-fold rise in blood level (G. F. Somers, quoted by Taussig, 1963). These data emphasize the role of species differences and illustrate the difficulties in predicting the teratogenic effects of a drug in man. There is general agreement that genetic variation during embryologic development conditions the development of congenital malformations by a drug. Thus, there are marked strain differences in production of cleft palate by cortisone in inbred mice (Fraser, Walker and Trasler, 1957). Differences in drug metabolism which may cause liberation of metabolites injurious to normal development is another mechanism. In random-bred species such as man, where the genotype of mother and fetus differ, aberrant drug metabolism in either mother or fetus must be considered. Such considerations may help to explain the failure of the characteristic malformations to occur in the offspring of all mothers who were exposed to a potentially teratogenic drug during the critical developmental stages.

*"Genocopy" of Thalidomide Embryopathy*

We have recently studied a large family with a dominant trait—the upper limb-cardiovascular syndrome—with some similarities to the thalidomide syndrome (Lewis, Bruce, Baum and Motulsky, 1963). The affected individuals presented with both upper limb and cardiovascular congenital malformations. The limb abnormalities ranged from hypoplastic thumbs to phocomelia. Cardiovascular abnormalities included transposition of great vessels leading to early death, absent left coronary artery, interarticular septal defect and others. Clinical manifestations varied between individuals, and no single patient had a clinical pattern similar to that of other affected family members. Chromosome studies showed a normal karyotype. The findings in this family seem to indicate that the mutant gene exerts its major action during differentiation of the upper limb and formation of the heart.

## Evolutionary Significance of Variation in Drug Response—Role of Foods

Many different catabolic reactions affecting drugs, such as oxidation

and hydroxylation, are carried out by a NADPH–requiring microsomal system in the liver of mammals. It has been suggested that this system represents an evolutionary adaptation to convert lipid-soluble organic food substances (such as alkaloids, terpenes, and steroids) into water-soluble substances in preparation for urinary excretion and may be considered as a relatively nonspecific protective mechanism (Brodie, Maickel and Jondorf, 1958). However, many other more specific biochemical reactions are also involved in drug metabolism.

Mammals and even lower organisms use broadly similar biochemical reactions to carry out key functions in metabolism. During evolution, variations in these reactions which do not compromise their major physiologic function may become established in a given species. Species differences in drug metabolism probably are based on this type of variation.

Intraspecies variation in a heterogeneous species such as man is likely to affect biochemical reactions just as it affects variation in external features. Analysis in depth of such variation is possible only if the enzymatic sequence involved can be investigated by both biochemical and genetic methods, such as has been achieved with pseudocholinesterase, glucose-6-phosphate dehydrogenase, and the acetylating enzyme concerned in INH metabolism.

The fairly high frequency of these variants in human populations raises the problem of their evolutionary origin. A selective advantage vis-à-vis an endemic infectious disease with high childhood mortality probably is a common mechanism leading to the establishment of a human polymorphism (Motulsky, 1960). There is in fact good evidence that G-6-Pd deficiency of the red cell protects against falciparum malaria (see above).

Differential mortality in famines which undoubtedly were common in the early days of man and his ancestors may be another mechanism by which polymorphisms have been established. Food substances have similarities to synthetic drugs, and variations in drug metabolism may owe their origin to differential food metabolism. Solanaceous food extracts are potent inhibitors of plasma cholinesterase (Harris and Robson, 1963). It is conceivable that the cholinesterase mutation causing resistance to enzyme inhibitors had a selective advantage under certain dietary conditions in the past. In that regard, the similarity of the gene frequencies of this trait in so many different populations is of interest.

The evolutionary origin of the INH acetylating polymorphism may involve acetylation of a food substance. Presence of the acetylating enzyme may have aided in detoxification of this unknown substance, which should be more common in the Asiatic diet, since the acetylating gene is most frequent among Asians. The inability to taste PTC is a recessively in-

herited trait. Many food substances share the chemical grouping (NC=S) which nontasters cannot taste. Again, foods may have played a role in the establishment of this polymorphism.

## Heterozygote Detection Using Drugs

### *Pentosuria*

In pentosuria there presumably is deficiency of the enzyme xylitol dehydrogenase. The enzyme is a catalyst in the glucuronic acid pathway of carbohydrate metabolism. Enzyme deficiency causes accumulation of the metabolite xylulose in serum and urine. When glucuronolactone or drugs such as aminopyrine which stimulate the glucuronic acid pathway are administered to pentosuric patients, markedly increased quantities of xylulose are excreted. Pentosuria is a recessive trait. Heterozygotes can be detected by increased xylulose in serum and urine after loading with glucuronolactone (Hiatt, 1958; Freedberg, Feingold and Hiatt, 1959).

### *Crigler-Najjar Syndrome*

Severe nonhemolytic jaundice often associated with kernicterus is found in this recessively inherited condition (Crigler and Najjar, 1952). The basic defect appears to affect the conjugation of bilirubin with glucuronides (Childs, Sidbury and Migeon, 1959). When drugs which normally undergo glucuronide-conjugation, such as salicylates, menthol, and tetrahydrocortisone, are administered to affected patients, decreased glucuronide conjugation is demonstrated. Heterozygotes who are not jaundiced can often be identified by defective salicylate conjugation (Childs et al., 1959).

## Drugs as Precipitants of Genetic Disease

Acute intermittent *porphyria* is caused by a dominant gene (for ref. see Goldberg, 1959). The trait may remain latent without ever showing clinical symptoms. Barbiturates have precipitated porphyria in latent cases and must be avoided by patients and family members who have been demonstrated to carry the gene. The frequency of paralytic complications in affected individuals is higher among those who took barbiturates during an attack of the disease (Goldberg, 1959). Presumably barbiturates affect the yet unknown basic lesion of this disease.

Phenothiazine may produce a *Parkinson-like syndrome* in some patients receiving this drug as a tranquilizer. The finding of a somewhat increased frequency of *Parkinsonism* among relatives of such patients (Myrianthopoulos, Kurland and Kurland, 1962) suggests a genetic predisposition to the condition.

Steroids and chlorothiazide may unmask latent *diabetes* (Goldner, Zarowitz and Akgun, 1960).

The recessively inherited trait acatalasemia was discovered when the oral mucosa of affected patients failed to show bubbles when hydrogen peroxide was applied (about 50 per cent of homozygotes have oral gangrene) (Takahara, 1952). This condition has been studied extensively and illustrates several genetic principles. The gene is very rare and appears restricted to Japanese populations, although it has been found in Switzerland (Aebi et al., 1962). Its distribution as studied by catalase assay, which detects heterozygotes, is spotty (Neel et al., 1963). Catalase deficiency can be used as a marked in tissue culture (Krooth et al., 1962). In the homozygous state no antigenically cross-reactive material can be found (Nishimura et al., 1962).

Pentothal anesthesia may cause severe respiratory depression in patients with mytonia dystrophia (Pachomov and Caughey, 1958).

## Detection of Genetic Heterogeneity by Drugs

A given disease may appear clinically uniform but may be caused by different genetic mechanisms. Depressive psychoses frequently run in families. Pare, Rees and Sainsbury, (1962) suggest that a given patient with depression will repeatedly respond favorably either to monoamine oxidase inhibitors or to imipramine drugs but not to both groups of drugs. They noted that sibs with depression responded to the same type of drug as the index case and suggest that response to monoamine oxidase inhibitors on the one hand and imipramine drugs on the other differentiates two genetically specific types of depression.

## Genetically Determined Drug Resistance

Resistance to a drug may have a simple genetic basis. Although human examples are not known, the genetics of atropine resistance in rabbits has been carefully worked out (Sawin and Glick, 1943). Rabbits may have a double dose, a single dose or no genes for the enzyme atropine esterase.

The level of enzyme on the average is more than twice as high in homozygotes as in heterozygotes. There is zero activity in homozygotes with no genes for the enzyme.

## Genetic Basis of Drug Allergy?

Drug allergies may be genetically determined. Evidence for heritability of human allergies and human autoimmune disease is good. Just as some strains of animals make antibodies more readily than others, the cellular capacity for antibody formation toward drugs may differ on a genetic basis. Another mechanism would postulate chemically altered drug metabolism causing uncovering of antigenically reactive groupings with formation of antibodies in occasional aberrant individuals.

## Chemical Mutagens in Man and Mammals

Many chemical substances have been shown to be mutagenic and chromosome breakers in micro-organisms and in plants (for ref. see Goldstein, 1962). Some of these substances such as caffeine and nicotine are widely used. Caffeine actually penetrates into the male gonad, but data for other potentially mutagenic agents and their penetration into male and female gonads are not available. If some of these substances as well as some commonly used drugs, such as nitrites, actually are mutagenic, chemical agents would be more important than radiation in raising the human mutation rate. Much more work needs to be done in this area. In one study (Lyon, Phillips and Searle, 1962) male and female mice were given drinking water containing 1 Gm. per l. of caffeine, up to 10 weeks of age. There was no difference in the age-specific locus mutation rate when 64,000 of their offspring were examined. This exposure to caffeine is approximately equivalent to a radiation exposure 25 r or less.

## Further Problems with Possible Genetic Implications

Many drugs exhibit variations. Further genetic investigations would appear fruitful in the following examples:

1) Phenylbutazone (Burns, 1962) and diphenylhydantoin disposal in man (Loeser, 1961) show marked variation and need family study.
2) Megaloblastic anemia develops occasionally with anticonvulsant ther-

apy. Aplastic anemia rarely develops with chloramphenicol therapy. Do these patients catabolize these drugs differently?

3) A lupus erythematosis-like syndrome develops occasionally in patients taking apresoline. Do these patients and their relatives have protein alterations predisposing them to this complication?
4) A bimodal response is noted in serum amylase when chlorothiazide is administered (Cornish, McClellan and Johnston, 1961). Is this response inherited and under control of a single gene?
5) There is individual variation in CNS stimulation by caffeine unrelated to caffeine blood levels (Goldstein, 1962). Is this a familial trait?

## Conclusion

Biochemical variability appears to be common in man. Drug reactions and deviant responses to drugs are an excellent screen for the detection of genetically determined enzyme abnormalities. Many advances in human and basic genetics have come from the study of such variants.

1. As in microbial genetics, a variety of different enzyme abnormalities within a given genetic system have been detected (i.e., pseudocholinesterase and glucose-6-phosphate dehydrogenase mutants). Some of these mutants may cause complete enzyme deficiency, others may lead to structurally altered enzyme and additional ones have not been fully defined.
2. Acetylation of different substances in man is carried out by more than one acetylating enzyme. Isoniazid acetylation is a polymorphic system with highest gene frequencies in the Orient.
3. The problem of dosage compensation for X-linked genes has been resolved for the G-6-Pd system and confirms the Lyon hypothesis.
4. The G-6-Pd system is an excellent model for the study of natural selection in human populations. The data indicate a protective effect of this trait vis-à-vis Falciparim malaria.
5. By stressing already altered biochemical reactions, drugs may precipitate or aggravate genetic disease. Examples include drug-induced hemolytic anemia, (G-6-Pd deficiency and hemoglobin Zurich), and barbiturates in porphyria. Similarly, drugs may be helpful in heterozygote detection.
6. Correlation analysis between groups of relatives suggest the operation of recessive genes in the metabolism of dicumarol in man.
7. Foods may have played a significant role in the establishment of variation affecting drug responses.

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Chapter 3

# Genetical Variants of Human Serum Pseudocholinesterase

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One criterion distinguishing animals from plants is the possession of cholinesterases, which have even been found in such primitive creatures as the Protozoa (Seaman and Houlihan, 1951). Cholinesterases are enzymes which hydrolyse cholinesters into free choline and the corresponding acid:

$$\underset{\text{ester of choline}}{R.COO.CH_2.CH_2.N^+(CH_3)_3} + H_2O \qquad R.COOH + \underset{\text{organic acid}}{} + \underset{\text{choline}}{HO.CH_2.CH_2.N^+(CH_3)_3}$$

As Alles and Hawes (1940) first showed, two different types of cholinesterase are found in man and other vertebrates: true cholinesterase and pseudocholinesterase. True cholinesterase metabolises acetylcholine with great rapidity, and a number of other cholinesters more slowly. It is found in high concentrations in nerve and muscle tissue, and in red cells, where it appears to be localized in the fibre membrane (Nachmansohn, 1959). The physiological role of true cholinesterase is the hydrolysis of acetylcholine at the neuromuscular junction and at some synapses. Nachmansohn (1959) has suggested that a similar mechanism may also be responsible for transmission in nerve and muscle tissues themselves.

Pseudocholinesterase exerts its maximum effect on longer chain cholinesters such as butyrylcholine, and splits acetylcholine relatively slowly. Specific substrates for both enzyme types exist: acetyl β-methyl choline is split only by true cholinesterase, and benzoylcholine only by pseudocholinesterase. Pseudocholinesterase is found in many tissues and in particularly high concentration in the serum, liver, the white matter of the brain, the heart, pancreas and skin. There is some evidence from animals that the pseudocholinesterases of different species differ in their substrate specificities (Davies, 1954). The term "pseudocholinesterase" may therefore include a heterogeneous family of enzymes. The physiological function of pseudocholinesterase is unknown. It is well known, however, that some cholinesters, and indeed acetylcholine itself in high concentration, are inhibitors of true cholinesterase and are hydrolyzed by pseudocholinesterase. Lehmann and Silk (1953) therefore suggested that pseudocholinesterase may protect the true enzyme against the inhibition produced by these compounds. These substances will be rapidly destroyed *in vivo* and would therefore be difficult to detect. But it is of interest that one such inhibitor, propionyl-choline, has been isolated from ox spleen by Banister, Whittaker and Wijesundera (1951).

The main points of differentiation between the two enzyme species are summarized in Table 1.

TABLE 1—*Comparison of the Properties of True Cholinesterase and Pseudocholinesterase*

| Property | True Cholinesterase | Pseudocholinesterase |
|---|---|---|
| Substrate range | Relatively small number of cholinesters | Large number of cholinesters and other esters such as benzoic acid derivatives |
| Optimum substrate | Acetylcholine | Longer chain, aliphatic acid esters of choline, *e.g.*, butyrylcholine |
| Specific substrate | Acetyl β-methylcholine | Benzoylcholine |
| Distribution in man | Nerve and muscle tissue; red cells | Liver, skin, white matter of brain, heart and pancreas; serum |
| Function | Destruction of the neuromuscular transmitter, acetylcholine | Not known; may protect true enzyme against inhibitory cholinesters |

*Action of Cholinesterase*

The concept, first proposed by Michaelis and Menten (1913), that enzymes exert their catalytic effect by a transitory combination with their substrate, has been well substantiated. A number of enzymes, of which the cholinesterases are an example, appear to possess two active sites, each of which combines with a different part of the substrate molecule. Detailed evidence in the case of the cholinesterases has been collected by Wilson and his colleagues (see Wilson, 1954). There is firstly the anionic site; this is a negatively charged region on the enzyme surface which combines with the positively charged nitrogen atom in the choline radicle. The second or esteratic site, combines with the substrate molecule at the ester linkage and is responsible for the ensuing hydrolysis. The theory is illustrated in Figure 1. The detailed evidence has been obtained from studies of true cholinesterase. The existence of two active sites on the pseudocholinesterase molecule is less certain, (see Augustinsson, 1960) although it seems probable that both occur at least in the case of the enzyme found in human serum.

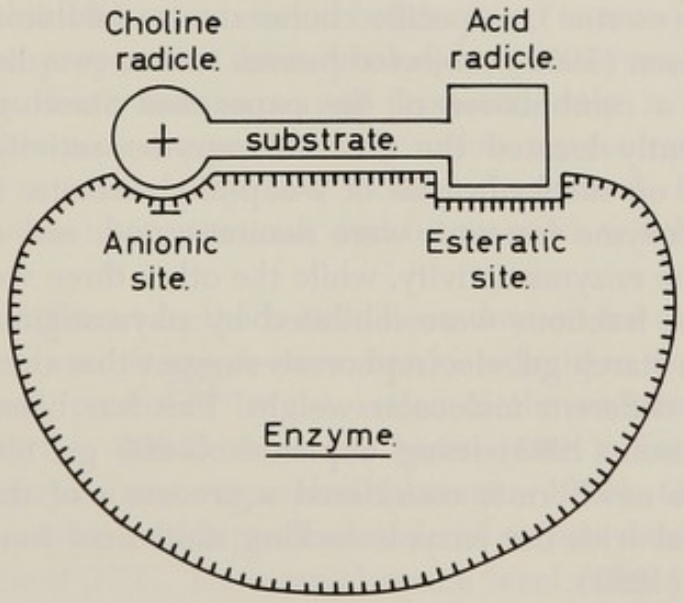


FIG. 1—The combination of cholinesterase with its substrate.

*Serum Pseudocholinesterase*

In human blood the distribution of the two enzymes is quite distinct: true cholinesterase is found in the red cells and pseudocholinesterase in the plasma.

The physical and chemical properties of pseudocholinesterase are not known in detail, largely because the enzyme has not been isolated in pure form. It is a protein with a molecular weight of about 300,000 (Surgenor and Ellis, 1954), and on electrophoresis–pH 8.6 migrates between the $\alpha_2$ and the $\beta$-globulins. A number of proteins in this group are glycoproteins, and Svensmark (1961) has shown that the pseudocholinesterase found in human serum is combined with sialic acid, a derivative of mannose. After sialic acid has been removed by incubation of serum with the enzyme sialidase, the electrophoretic mobility of pseudocholinesterase is so reduced that it migrates with the $\gamma$-globulins. The enzymatic activity of the pseudocholinesterase is however unaffected by this change. The detailed findings suggest that several molecules of sialic acid are attached to one molecule of pseudocholinesterase and that the points of attachment are not at the active sites of the enzyme.

There is some evidence that serum pseudocholinesterase is heterogeneous. Dubbs and his colleagues (1960) have subjected human serum to starch gel electrophoresis followed by staining of the starch block with a variety of enzyme substrates. There were two narrow bands of enzyme activity in the $\alpha$-$\beta$-globulin position both of which gave the staining reaction for pseudocholinesterase, and both of which were inhibited by physostigmine (eserine), a specific cholinesterase inhibitor. Harris, Hopkinson and Robson (1962) subjected human sera to two-dimensional electrophoresis by a combination of the paper and starch-gel techniques. They subsequently located the areas of enzyme activity with a stain which depends on the hydrolysis of $\alpha$-naphthyl acetate. By this means, four separate enzyme fractions were demonstrated, one comprising the major part of the enzyme activity, while the other three were minor components. All the fractions were inhibited by physostigmine. The different mobilites in starch gel electrophoresis suggest that these four enzyme fractions an of different molecular weight. This has been confirmed by Harris and Robson (1963) using 'Sephadex G-200' gel filtration. A fraction seen in the newborn is considered a precussor of the enzyme, and may be identical with the enzyme lacking sialic acid found in the liver by Svensmark (1963).

It is not yet possible to relate histochemical findings to other work on pseudocholinesterase with complete confidence. Naphthyl acetate is hydrolyzed by esterases other than the pseudocholinesterases. On the other hand, the esterases demonstrated by Harris, Hopkinson and Robson (1962) were all inhibited by physostigmine–and this is considered typical for pseudocholinesterase. Also a serum from a woman with genetically determined absence of pseudocholinesterase activity (Liddell, Lehmann and Silk, 1962) produced in our hands no stained fraction on electro-

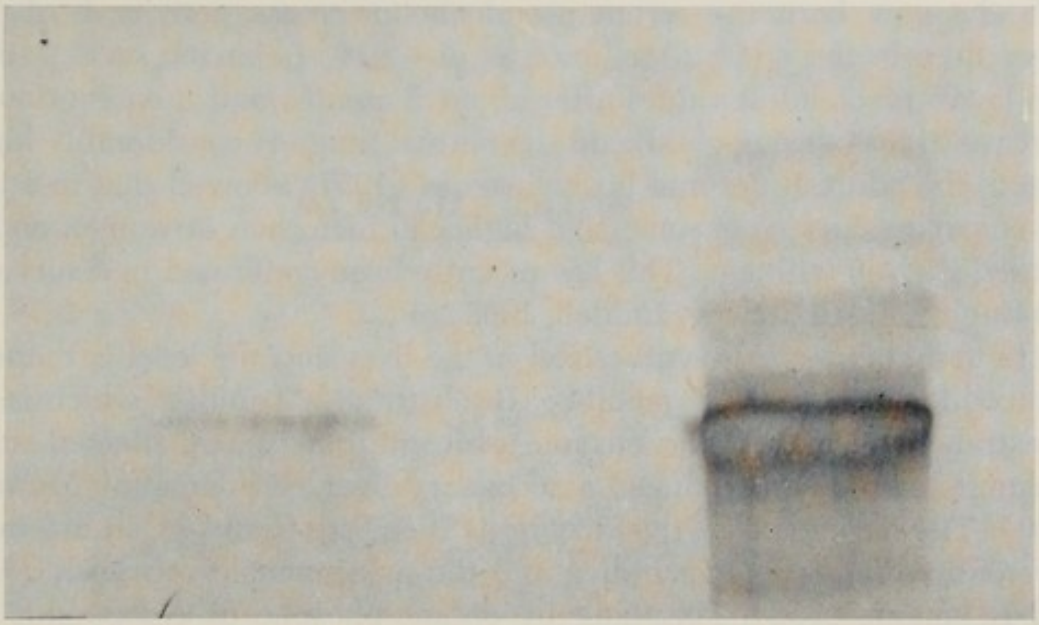

FIG. 2—Starch gel electrophoresis according to Poulik (1957) stained for esterase. Left: Pseudocholinesterase concentrate (Cutter Laboratories). Right: Fresh serum from a normal homozygote. It will be seen that with Pintér's technique (1957), 10 fractions can be demonstrated in the concentrate, but only one in fresh serum.

phoresis. The results of electrophoretic separation are shown in Figure 2. It can be seen that 10 fractions could be identified in a concentrated pseudocholinesterase preparation from pooled human serum, yet under the same conditions fresh human serum produced only a single fraction. Possibly the other nine are present only in low concentration and fail to reveal themselves unless they have been concentrated. However, there are several possible interpretations, and future work will have to provide the answer.

A number of different methods have been devised for estimating serum pseudocholinesterase. A micro-manometric technique is often used in which acetic acid, produced by the action of the enzyme on acetylcholine, liberates carbon dioxide from the bicarbonate buffer in which both substrate and enzyme are suspended (Ammon, 1933). One unit is that amount of the enzyme which produces 1 L. of carbon dioxide in 1 minute at a temperature of 37°C. The normal serum level ranges from 60 to 125 units/ml. Another method, devised by Kalow and Lindsay (1955) depends on the fact that benzoylcholine, a specific substrate for the enzyme, absorbs ultraviolet light strongly at a wave length of 240 mu, while the products of its hydrolysis do not show this absorbance. The rate of decrease of this absorbance when serum and enzyme are mixed can therefore be measured in a spectrophotometer. One unit is defined as the hydrolysis of 1 M of benzoylcholine per hour at a temperature of 25°C. The normal range is 30 to 120 units/ml.

In the new born the serum pseudocholinesterase activity is slightly lower than in the adult (McCance et al., 1949; Lehmann et al., 1957). The levels reach adult values after about 2 months and then continue to increase so that during childhood the normal range is considerably higher than in the adult. Rider and his colleagues (1957) showed that in adults, the enzyme values were somewhat higher in men than in women and increased slightly with age. This has recently been confirmed in a survey of 500 English blood donors (Liddell, 1962).

The serum enzyme is synthesized in the liver and the level is therefore reduced in hepatocellular jaundice. In obstructive jaundice which is due to extrahepatic causes, the enzyme concentration is not affected unless prolonged stasis has produced a secondary liver cell damage (McArdle, 1940). The estimation of the enzyme is therefore useful as an aid in the differential diagnosis of jaundice and the assessment of cirrhosis (Hunt and Lehmann, 1960). Poisoning by organo-phosphorus compounds also depresses the serum activity. These cholinesterase inhibitors are now widely used as agricultural insecticides, and cases of accidental poisoning have become relatively common (Namba and Hiraki, 1958). Other pathological conditions associated with a slighter and more inconstant lowering of the enzyme activity are malnutrition, hyperpyrexia, cardiac failure, uremia and catatonia (Antopol, Tuchman and Schifrin, 1937; McArdle, 1940; Tod and Jones, 1937). Increased levels of serum pseudocholinesterase have been reported in obesity (Berry, Cowin and Davies, 1954), nodular goiter (Koster and Kisch, 1943) and nephrosis (Kunkel and Ward, 1947).

Pseudocholinesterase is also of clinical importance because it inactivates a number of drugs which are commonly used in anesthesia. The majority of these are local anesthetics such as procaine, 2 chloro-procaine and amethocaine. These drugs are all esters of benzoic acid derivatives and are hydrolyzed by pseudocholinesterase (Legge and Durie, 1942; Kalow, 1952).

Another drug detoxified by the serum enzyme is the short-acting muscle relaxant, suxamethonium. Although some of the pharmacological actions of this compound were noted as early as 1906 by Hunt and Taveaux, the muscle relaxant properties were first described by Bovet and his colleagues (1949). This compound, often also called succinyldicholine or succinylcholine, (proprietary names scoline and anectine) is the dicholine ester of succinic acid, and Figure 3 shows that it can be regarded as consisting of 2 acetylcholine radicles joined by their acetate groups. It is therefore not surprising that the drug is rapidly hydrolyzed by serum pseudocholinesterase. This was first shown by Glick (1941) before the muscle relaxant properties of suxamethonium were appreciated, and independently con-

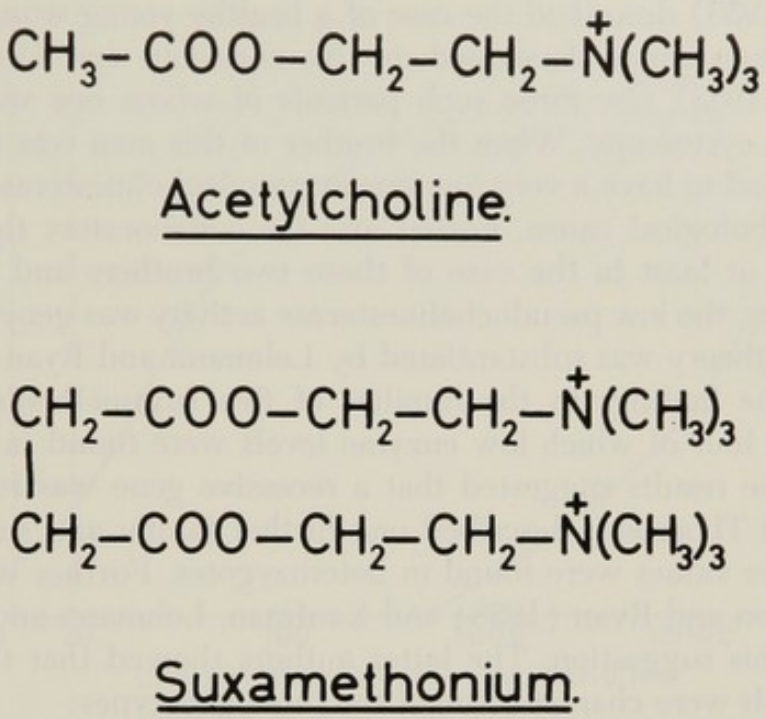


FIG. 3—Comparison of the formula of suxamethonium with that of acetylcholine.

firmed by Bovet-Nitti (1949). Whittaker and Wisejundera (1952), using horse serum as the source of the enzyme, found that the hydrolysis takes place in two stages with succinyl monocholine formed as an intermediate, while Lehmann and Silk (1953) showed that this was also true of the human enzyme.

The rapid hydrolysis of the drug is responsible for its short action which has proved so convenient a property in anesthesia. After the usual therapeutic dose of between 30 and 100 mg., the muscular paralysis and associated apnea last only about 2 or 3 minutes. So useful is the drug that in England the majority of patients who undergo an operation with general anesthesia are given suxamethonium. In addition, the drug is widely used as an adjunct to electroconvulsive therapy for psychiatric disorders and in minor orthopedic manipulation. It is also given in a drip infusion as a treatment for tetanus.

*Inheritance of Low Enzyme Levels*

Shortly after the introduction of suxamethonium a few cases were described of prolonged apnea, lasting in some patients for several hours, and Bourne, Collier and Somers (1952) and Evans, Gray, Lehmann and Silk (1952) showed that such patients had a low serum pseudocholinesterase level. As more of these rare individuals were discovered it was realized that the low enzyme values were not always due to pathological causes such as liver disease.

Bourne (1953) described the case of a healthy young woman in whom suxamethonium apnea developed on two occasions and Forbat and his colleagues (1953) saw three such patients of whom one was a Cypriot subjected to cystoscopy. When the brother of this man was examined he also was found to have a very low serum pseudocholinesterase level without any pathological cause. Forbat and his collaborators therefore suggested that, at least in the case of these two brothers and possibly the other patients, the low pseudocholinesterase activity was genetically determined. The theory was substantiated by Lehmann and Ryan (1956) who described the findings in the families of five suxamethonium sensitive propositi, in four of which low enzyme levels were found in some of the relatives. The results suggested that a recessive gene was responsible.

Allott and Thompson described one further family and suggested that slightly lower values were found in heterozygotes. Further work by Lehmann, Patston and Ryan (1958) and Kaufman, Lehmann and Silk (1960) supported this suggestion. The latter authors showed that the following enzyme levels were characteristic of the three genotypes:

Normal homozygotes: greater than 60 units/ml.

Heterozygotes: between 26 and 90 units/ml.

Abnormal homozygotes: less than 35 units/ml.

It can be seen that the heterozygote values overlap considerably with those found in both types of homozygote. Because of this it is often impossible to determine an individual's genotype with certainty by enzyme estimations alone. For instance, an individual with an enzyme level of 70 units/ml. could be either a normal homozygote or a heterozygote. These difficulties have been resolved by the work of Kalow and his colleagues in Canada.

*Kalow's Concept of Atypical Pseudocholinesterase*

Kalow and his collaborators have shown that the low enzyme activity found in the serum of an abnormal homozygote is due, not to a low concentration of the normal enzyme, but to an unusual variant of this enzyme which is less effective in hydrolyzing substrates (Kalow and Genest, 1957; Kalow and Staron, 1957; Kalow and Davies, 1958; Davies, Marton and Kalow, 1960). The two enzymes differ in the following respects. Firstly the atypical enzyme hydrolyses all substrates more slowly than the usual variant, *i.e.*, it has a higher Michaelis constant (Davies, Marton and Kalow, 1960). This is particularly marked when suxamethonium is the substrate. Figure 4 shows the effect of varying concentrations of this substrate on the speed of hydrolysis by the two enzyme variants. It can be seen that at the concentrations to be expected in the anesthetized subject,

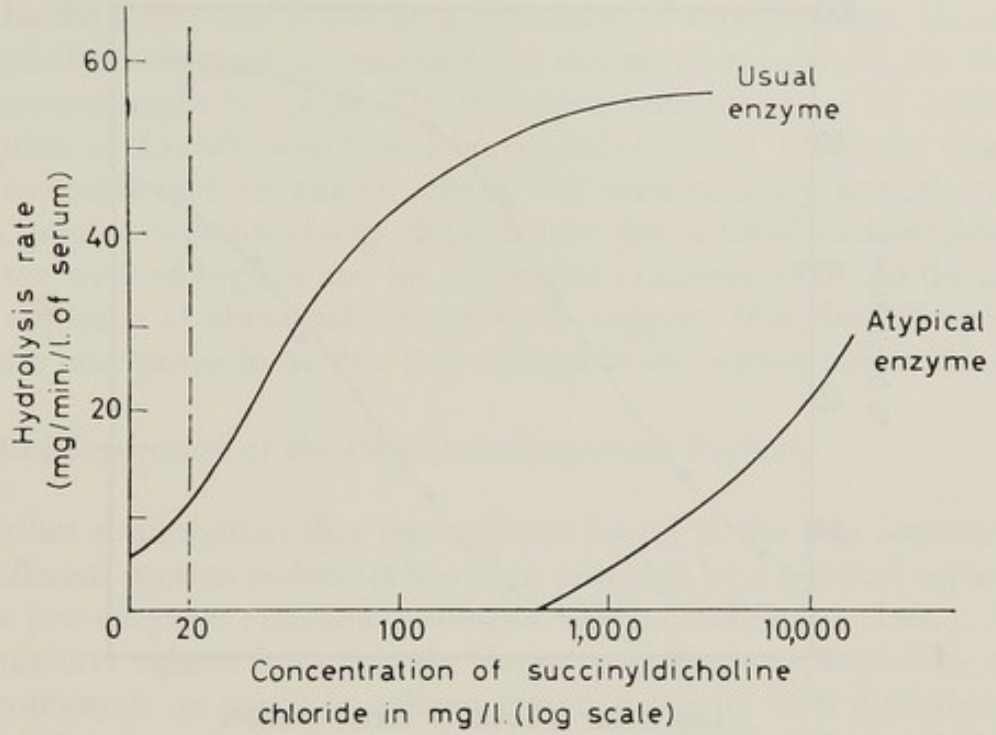


FIG. 4—The different rates of hydrolysis of suxamethonium by the pseudocholinesterase variants (after Kalow, 1960). The dotted line indicates the concentration found *in vivo*.

the atypical enzyme has no detectable effect on suxamethonium.

The second distinction between the two enzyme variants is that the unusual enzyme is more resistant to the majority of pseudocholinesterase inhibitors (Kalow and Davies, 1958). A characteristic shared by all these differential inhibitors is that they possess a positively charged nitrogen atom in the molecule. A smaller group of inhibitors, the organo-phosporus compounds, do not contain such a positively charged radicle, and inhibit both enzymes equally.

The local anesthetic dibucaine (cinchocaine) is one of the differential inhibitors. Figure 5 shows the inhibitory effect of varying concentrations of this substance on the two enzymes when benzoylcholine is the substrate and other conditions are standardized. The distinction between the two pseudocholinesterases is most marked when the dibucaine concentration is $10^{-5}$ molar: the normal enzyme is inhibited by about 80 per cent and the unusual variant by only about 20 per cent. Kalow and Genest (1957) termed the percentage inhibition produced by this concentration of dibucaine, the "dibucaine number" or "DN." The dibucaine number is independent of the enzyme concentration, and, remains at about 80 per cent in the normal homozygote even when disease has reduced the activity to low levels usually found in the abnormal homozygote. When sera from

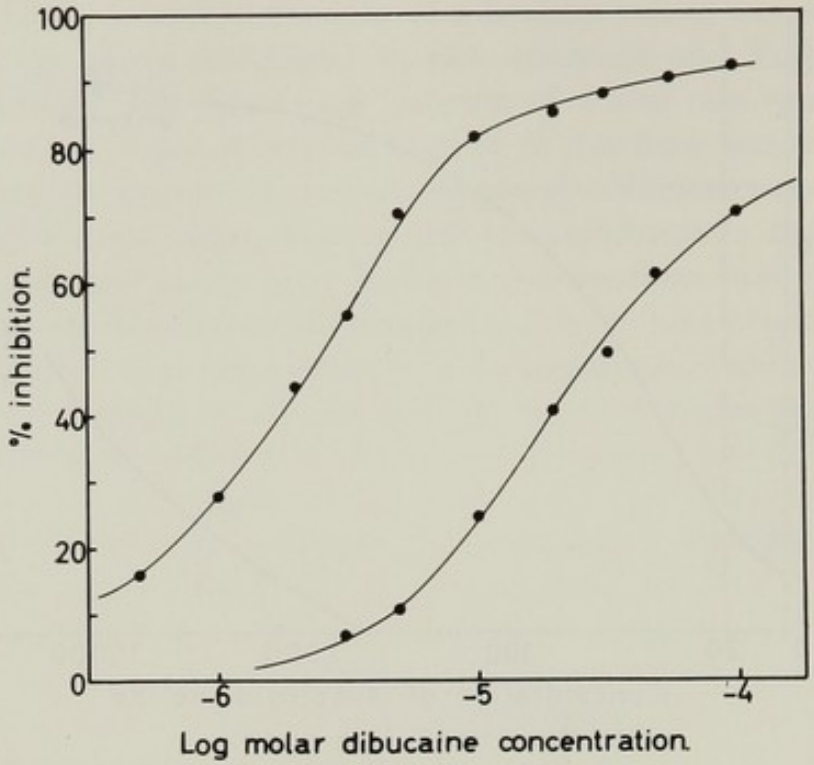


FIG. 5—The inhibitory effect of varying concentrations of dibucaine on the pseudocholinesterase variant (after Kalow and Genest, 1957).

those individuals whom family studies suggest are heterozygous are examined, the dibucaine numbers are found to lie between 52 and 69 per cent. These results, unlike those obtained from the estimation of enzyme activity, do not overlap with the values found in both types of homozygote. The genotype of an individual can therefore be determined with certainty by this technique.

Another differential inhibitor is the dimethyl carbamate of (2 hydroxy-5-phenylbenzyl) trimethyl ammonium bromide, more conveniently known by the manufacturers code name, RO2-0683. A $10^{-8}$ molar concentration inhibits the normal enzyme by over 90 per cent and the unusual variant by less than 10 per cent. Although the differentiation is even greater than with dibucaine, this substance does have the disadvantage of requiring preincubation with the enzyme.

Among the positively charged inhibitors, the differential effect on the two pseudocholinesterases is varied: the more potent an inhibitor the greater is the difference in its effect on the two enzymes. This suggests that we are dealing with a real difference in the enzyme molecule itself. It was theoretically possible that these differences in activity were due not to changes in the enzyme, but to a competitive inhibitor of pseudocholinesterase, whose presence is genetically determined. But, as Kalow and Davies (1958) pointed out, the differential effect of all inhibitors would

then be the same, and would be independent of their potency. Moreover, it is possible to suggest at what point on the enzyme molecule, the change in structure might be found. The differential inhibitors are all (with the exception of fluoride which will be described later) positively charged. This indicates that the anionic site on the more sensitive normal enzyme has a greater negative charge than that on the unusual variant. Analogy with the work of Ingram and his colleagues (Ingram, 1957) on the amino acid sequence of abnormal hemoglobins, suggests that the difference at this site may prove to be due to a change in one amino acid residue.

*Physical Separation of the Pseudocholinesterase Variants*

Further confirmation that the enzymes found in the two homozygotes are different protein molecules has been provided by a physical separation of the two enzymes (Liddell, Lehmann, Davies and Sharih, 1962). Artificial mixtures of sera from the two homozygotes were separated by either electrophoresis on paper or column chromatography with diethyl-aminoethyl cellulose as the anionic exchange material. With either technique it was possible to separate two different fractions, both with pseudocholinesterase activity; one of which had a dibucaine number characteristic of the abnormal homozygote and the other one typical of the normal homozygote. This provides unequivocal evidence that the biochemical differences are due to changes in the enzyme molecule and not to the presence or absence of modifying substances in the sera. The results are also compatible with the theory that the anionic site possesses a greater negative charge on the normal enzyme than on the atypical variant: at alkaline pH the normal enzyme moved more rapidly towards the anode on paper electrophoresis and was eluted from a cellulose column with greater difficulty than the unusual pseudocholinesterase.

Sera from heterozygotes were fractionated by the same techniques. While the intermediate dibucaine values of sera from this genotype had suggested that these individuals possessed a simple mixture of the two enzymes, other explanations were possible. For instance, Smithies and his colleagues have shown that the heterozygote for the haptoglobin alleles possesses a mixture of haptoglobin proteins which are not found in either homozygote (Smithies and Connell, 1955). It appears probable that this is due to polymer formation between the polypeptide subunits which are the primary gene products. Some enzymes are also known to be formed from polypeptide subunits: $\alpha$-chymotrypsin and rabbit muscle aldolase (Dreyer et al., 1955; Rutter, 1961). It was therefore possible, to envisage that the heterozygote for pseudocholinesterase possessed one enzyme, formed from two different polypeptide chains, and with properties inter-

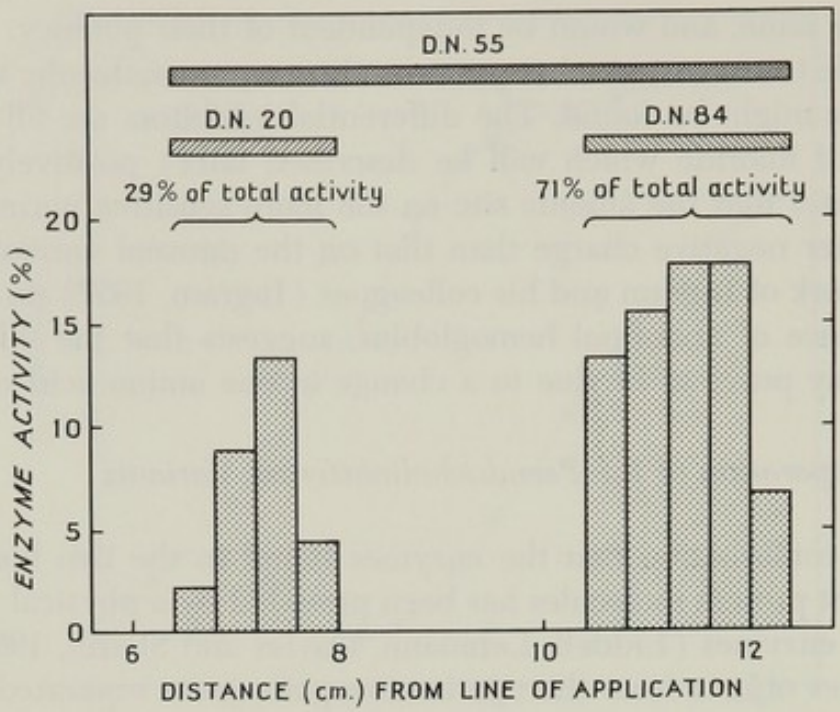


FIG. 6—Pseudocholinesterase activity in eluates obtained from 0.4 cm. strips cut serially after paper electrophoresis of serum from a presumed heterozygote (D.N. = 55). In this particular experiment the albumin was allowed to run off the paper-strip into the buffer (from Liddell, Lehmann, Davies and Sharih, 1962).

mediate between those of the two homozygotes. A more complex series of intermediate proteins was also possible.

However with both techniques, two pseudocholinesterase fractions were obtained from heterozygous sera; one with a dibucaine number of about 20 per cent and the other of about 80 per cent (Fig. 6 and 7). It can therefore be concluded that the heterozygote possesses a simple mixture of the two enzymes.

*Genetic Variants in Other Tissues*

Pseudocholinesterase activity has been described in many tissues. Liddell, Newman and Brown (1963) obtained postmortem specimens of brain, liver, kidney, small intestine and skin from subjects who had previously been shown, by family studies and examination of their sera, to be an atypical homozygote and a heterozygote, respectively. Dibucaine numbers and the percentage inhibition to RO2-0683 were estimated on the pseudocholinesterase activity found in soluble extracts of these tissues. Specimens from five normal homozygotes were also examined. In each case the values were the same as those found with the serum of the same subject.

It therefore appears that the genetic variant of pseudocholinesterase is

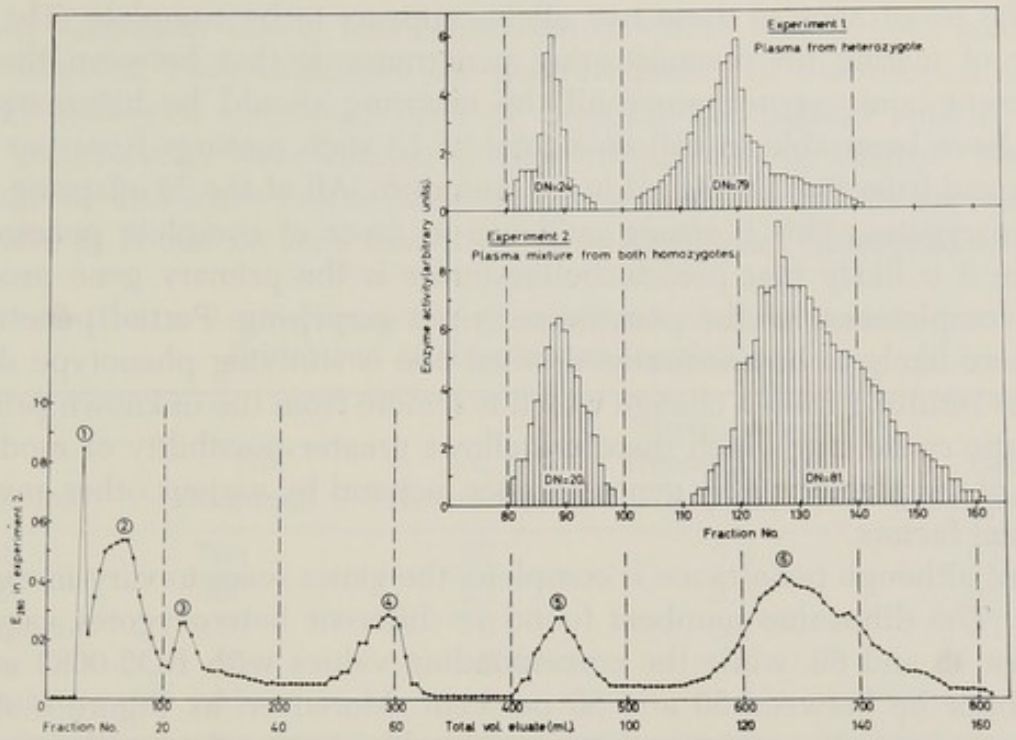


FIG. 7—Fractionation of plasma by column chromatography on DEAE cellulose and elution by increasing concentrations of disodium hydrogen phosphate. Top row: Pseudocholinesterase fractions obtained from the plasma of a presumed heterozygote. Middle row: Pseudocholinesterase fractions obtained from a mixture of plasma of two homozygotes. Bottom row: The protein concentration of the fractions obtained in the second experiment (as measured by the absorption of ultraviolet light). Fraction 1 and 2 contain the $\gamma$-globulins, and fraction 6 the albumin. It will be seen that by this method the usual pseudocholinesterase is eluted with the albumin.

found in these tissues as well as in the serum. This implies that the same gene determines the production of both the tissue and the serum enzymes, and that the primary structure of the protein molecule is the same in each case. It is, of course, possible that other human pseudocholinesterases exist, either in tissues which were not examined by the authors, or in a form which is not rendered soluble by the techniques employed.

*The Mode of Inheritance and Gene Frequencies*

Family studies show that the inheritance can be explained by the presence of two co-dominant allelic genes. For instance, in 39 out of 41 families investigated by the present authors, the inheritance was compatible with this theory. The two exceptions were due to the presence of yet another allelic gene which will be discussed later. Similar evidence has been published by Kalow and Staron (1957), Harris, Whittaker, Lehmann and Silk (1960) and Bush (1961).

The penetrance of these two alleles appears to be complete. The best type of mating for demonstrating penetrance is that between the two different homozygotes, since all the offspring should be heterozygotes. We have been able to collect a total of 13 such matings from our own data and from that published in the literature. All of the 29 offspring were heterozygotes. This is strong evidence in favor of complete penetrance. Since it is likely that pseudocholinesterase is the primary gene product, the completeness of the penetrance is not surprising. Partial penetrance is more likely to be encountered when one is studying phenotype differences resulting from a change which is remote from the unknown primary genetic cause and which therefore allows greater possibility of modification of the phenotype by genes at other loci and by various other environmental factors.

But although penetrance is complete, the genes seem to vary in expression. The dibucaine numbers found in different heterozygotes vary between 48 and 69, while the corresponding values with RO2-0683 as the inhibitor lie between 50 and 80 per cent. Moreover, as Figure 8 shows there is a rough correlation between the dibucaine values and those obtained using RO2-0683. This suggests that both values are an expression of the same variable, namely, the relative concentrations of the two enzymes.

The gene frequencies have now been determined in a number of populations. Because of the rarity of the unusual homozygote this has been done by surveys to detect the heterozygotes by the biochemical techniques using dibucaine and RO2-0683. Kalow and Gunn (1958) surveyed 2,017

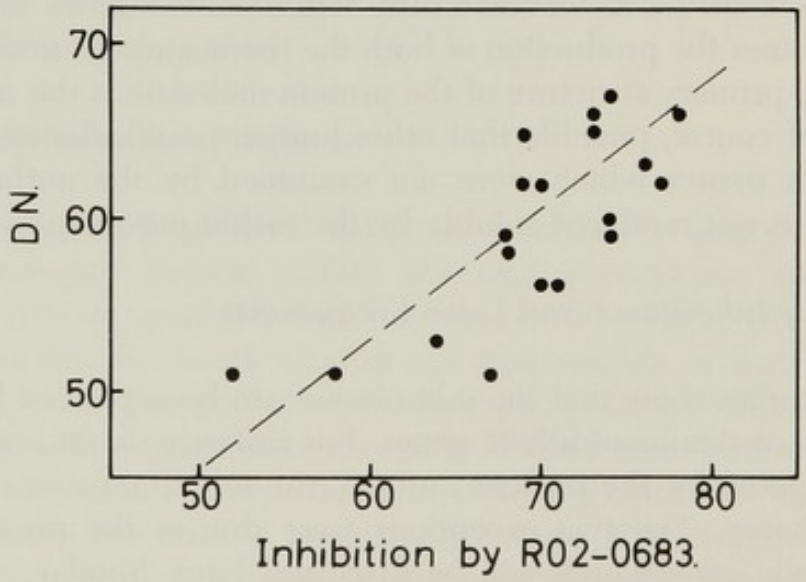


FIG. 8—The relationship between the dibucaine number and the percentage inhibition produced by RO2-0683 as seen in the plasma of 20 heterozygotes.

healthy Canadian adults and found one atypical homozygote, and 74 heterozygotes. They calculated the gene frequency of the unusual allele to be 0.0198 with a standard error of ±0.0022. The present authors and their collaborators have compared gene frequencies in English, Greek, Portuguese and some North African populations. The results are shown in Table 2. It can be seen that there is no significant difference among

TABLE 2—*Frequency of Atypical Pseudocholinesterase in British and Mediterranean Populations*

| Population | No. Examined | No. of Heterozygotes | Frequency of Heterozygotes (%) | Gene Frequency |
|---|---|---|---|---|
| British | 703 | 27 | 3.8 | 0.019 |
| Greek | 360 | 13 | 3.6 | 0.018 |
| Portuguese | 179 | 6 | 3.4 | 0.017 |
| Berber | 55 | 2 | 4 | – |
| Moroccan-Jewish | 51 | 1 | 2 | – |

After Kattamis et al. (1962).

these populations. Goedde and Altland (1963) found a closely similar frequency in 200 individuals from Prague + Southern Germany, while Horsfall, Lehmann and Davies (1963) found one heterozygote among 104 Australian aboriginals. Although a smaller frequency was found in the latter survey, the difference is not statistically significant. A similar survey is in progress on various Asiatic populations, one of them with Dr. Lielnjo Luan Eng of the Medical Research Institute, Kuala Lumpur, Malaya. The results are not complete, but suggest that the gene frequencies are similar. These findings are rather surprising when such considerable differences in the gene frequencies among different ethnic groups have been found in blood groups, abnormal hemoglobins and haptoglobins.

The frequency of abnormal homozygotes calculated from the gene frequency is 1 in 2,800 with a standard error ranging from 1 in 2,300 to 1 in 3,650 (Kalow and Gunn, 1958). A reliable direct estimate of the frequency of this genotype has not been made. Because of their rarity, it is practicable only to assess the frequency of these individuals by the incidence of suxamethonium apnea. In this way, Lehmann and Ryan (1956) predicted that the frequency of this rare genotype must be less than 1 in 1,000. Such an estimate may involve many errors. The causes of apnea during anesthesia are complex, and apparent cases of suxamethonium sensitivity must therefore be shown to be abnormal homozygotes by biochemical investigation of sera from their relatives and themselves. Such

an investigation will prevent an overestimate of the frequency being made. But several less easily analyzed factors may result in an underestimate of the frequency. For instance, we have seen one patient, an abnormal homozygote who had a 90 minute period of apnea following suxamethonium given during a minor operation. During a previous operation she had shown only a 10 minute period of apnea following the same dose of suxamethonium. This was so slight that the anesthetist had attributed the apnea to other causes and she had not been investigated. During the first operation she was receiving a blood transfusion, and this must have cut short the apnea as pseudocholinesterase keeps well in blood stored for transfusion purposes. Indeed, we believe that a transfusion of blood or plasma is the best treatment for suxamethonium apnea (Lehmann, 1952). Since blood transfusions are frequently given during surgery, some abnormal homozygotes may not be recognized by their response to suxamethonium.

We have recently attempted to determine the incidence by this method. In a group of six hospitals served by one of us (J. L.) there have been nine cases of suxamethonium apnea during a 2 year period. Biochemical investigation of these individuals and their families have shown that six of these were homozygotes for the unusual pseudocholinesterase allele. During this period approximately 20,670 patients were given suxamethonium. These figures give an incidence of abnormal homozygotes of 1 in 3,440. Although the figures can be approximate only, the rough agreement between the direct estimate and that calculated from the heterozygote frequency does provide further evidence that the genetic theory is correct.

The pseudocholinesterase variants may have some value in forensic medicine. In cases where paternity is disputed the complete penetrance shown by the unusual allele would be of value, although the rather low frequency of heterozygotes would limit the application of the test. The unusual stability of the enzyme is an advantage in forensic work. Keilin and Wang (1947) have shown that about 40 per cent of the cholinesterase activity had survived in a sample of horse blood which had been stored in the dark at room temperature for 25 years. Lehmann and Davies (1962) have shown that dibucaine numbers can be determined on specimens obtained from very small blood stains, some of them several years old.

*Unusual Types of Inheritance*

While the majority of data confirm the simple Mendelian inheritance, a few families have been reported in which the findings are not com-

patible with this theory. One such family has been described by Lehmann, Silk, Harris and Whittaker (1960). The dibucaine numbers of the father and mother suggested that they were a heterozygote and a normal homozygote, respectively. One child appeared to be a normal homozygote, while the other five had enzyme levels characteristic of the abnormal homozygote, and dibucaine numbers intermediate between those found in abnormal homozygotes and in heterozygotes. Interpretations of these anomalous findings cannot be given until further families of this type are discovered.

*Fluoride Inhibition*

Harris and Whittaker (1961) have shown that sodium fluoride is a differential inhibitor of the serum pseudocholinesterase variants, and that the three genotypes can be identified by means of this inhibitor in a manner similar to the dibucaine technique. They have termed the percentage inhibition produced when $5 \times 10^{-5}$ molar sodium fluoride is used instead of $10^{-5}$ molar dibucaine, the fluoride number or F.N. This in itself is an interesting observation since all other differential inhibitors of the pseudocholinesterase variants are positively charged. The finding is not necessarily incompatible with the theory that the unusual variant is due to a point mutation at the anionic site of the enzyme. Fluoride may have an indirect effect on substrate-enzyme binding through its effect on calcium or magnesium ions. Harris and Whittaker (1963) compared the effect of fluoride with that of chloride. Sodium chloride also acts as an inhibitor but at a very much higher concentration (0.6 molar), and then inhibits the atypical enzyme more strongly than the usual variant.

Harris and Whittaker (1961) have studied a series of families using both the dibucaine and the fluoride techniques. In the majority of these families, individuals were assigned to the same genotype by both inhibitors. In a few families, individuals apparently belonging to either the normal or the heterozygous genotype were found with the following characteristics: (1) Dibucaine numbers which, while within the ranges found in other individuals of their apparent genotype, were near the lower limits of those ranges; (2) fluoride numbers which were considerably lower than those usually found in individuals of the same apparent genotype. These findings suggest that a further variant of the normal enzyme exists, with a slightly decreased sensitivity to dibucaine and a markedly decreased sensitivity to fluoride.

Harris and Whittaker (1962a) confirmed this theory in a study of 22 further families. In two of these families the mating was between a normal homozygote and an individual with a dibucaine number character-

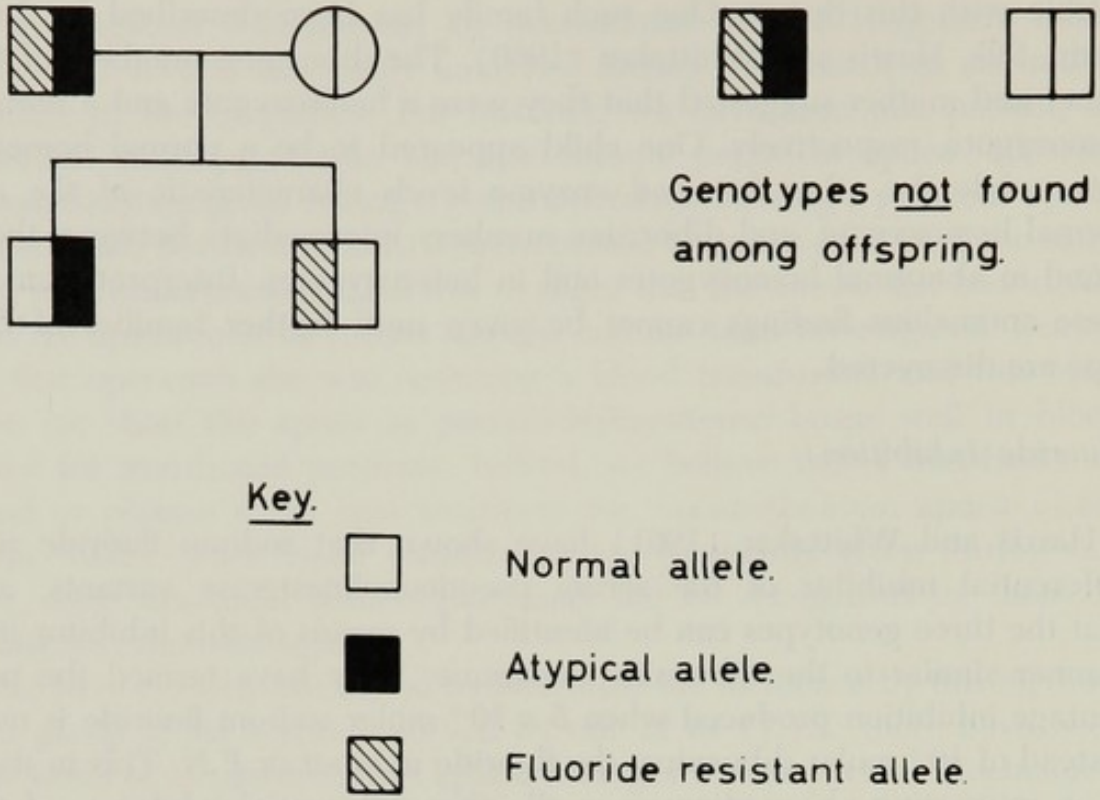


FIG. 9—The genotypes found among the offspring when one parent appeared to be a normal homozygote and the other a heterozygote for both the atypical allele and that for the fluoride resistant enzyme.

istic of the heterozygote but with a reduced fluoride number. The latter individuals should therefore be heterozygous for the atypical allele and for that determining a decreased sensitivity to fluoride. Of the 10 children, five were heterozygotes with the response to fluoride usually found in that group, and five had normal dibucaine numbers, but low fluoride numbers. Thus the determinant for the low fluoride number always segregated with the normal dibucaine allele and never with that determining low dibucaine sensitivity. This is illustrated in Figure 9. Harris and Whittaker point out that although their data are as yet insufficient to exclude the presence of a modifying gene at another locus, this possibility appears remote.

We have seen four families in whom this gene appears to be segregating (Lehmann, Liddell, Blackwell, O'Connor and Daws, 1963; Liddell, Lehmann and Davies, 1963). In all cases the propositi were discovered because they showed a moderate sensitivity to suxamethonium. In three families, examination of the sera suggested that the propositi were heterozygotes possessing both the atypical gene and that determining fluoride resistance. In these families also, the findings were compatible with the

theory that the gene for fluoride resistance was an allele of the other pseudocholinesterase genes (Table 3).

TABLE 3—*Phenotypes of Offspring*

| | Normal Homozygote | Heterozygote Normal/ Fluoride-Resistant | Heterozygote Normal/ Dibucaine-Resistant | Heterozygote Fluoride-Resistant Dibucaine-Resistant |
|---|---|---|---|---|
| *Expected* | | | | |
| Allele theory | Nil | 50% | 50% | Nil |
| Two loci theory | 25% | 25% | 25% | 25% |
| *Observed* | | | | |
| No. of matings | | | | |
| Harris and Whittaker (1962) 2 | — | 5 | 5 | — |
| Liddell, Lehmann and Davies (1963) 2 | — | 3 | 3 | — |
| Unpublished data 1 | — | 1 | 1 | — |
| Total | — | 9 | 9 | — |

Are the usual, the unusual and the fluoride resistant psuedocholinesterase genes alleles? Or is more than one locus involved? The offspring of the mating between a normal homozygote and a heterozygote for the genes determining the atypical (dibucaine resistant) and fluoride resistant enzymes.

In the fourth family both the suxamethonium sensitive propositus and one sibling appeared to be homozygotes for the fluoride-resistant gene. The dibucaine and fluoride numbers of these individuals and the apparent genotype of their relatives all supported this hypothesis. Moreover, when the serum of the propositus was fractionated by paper electrophoresis, the pseudocholinesterase appeared to be homogeneous. On the other hand, a similar experiment on one of the propositi of the first three families showed a partial separation of the atypical enzyme from the fluoride-resistant variant.

The use of dibucaine and fluoride as differential inhibitors now permits the recognition of six genotypes.

The findings in these are illustrated in Figure 10. The values found in the heterozygotes should lie on the straight lines joining the values for the three homozygotes. They need not lie at the midpoint of these lines, because even if the molecules of the enzyme variants are produced in equal numbers, they may have different enzymic activities.

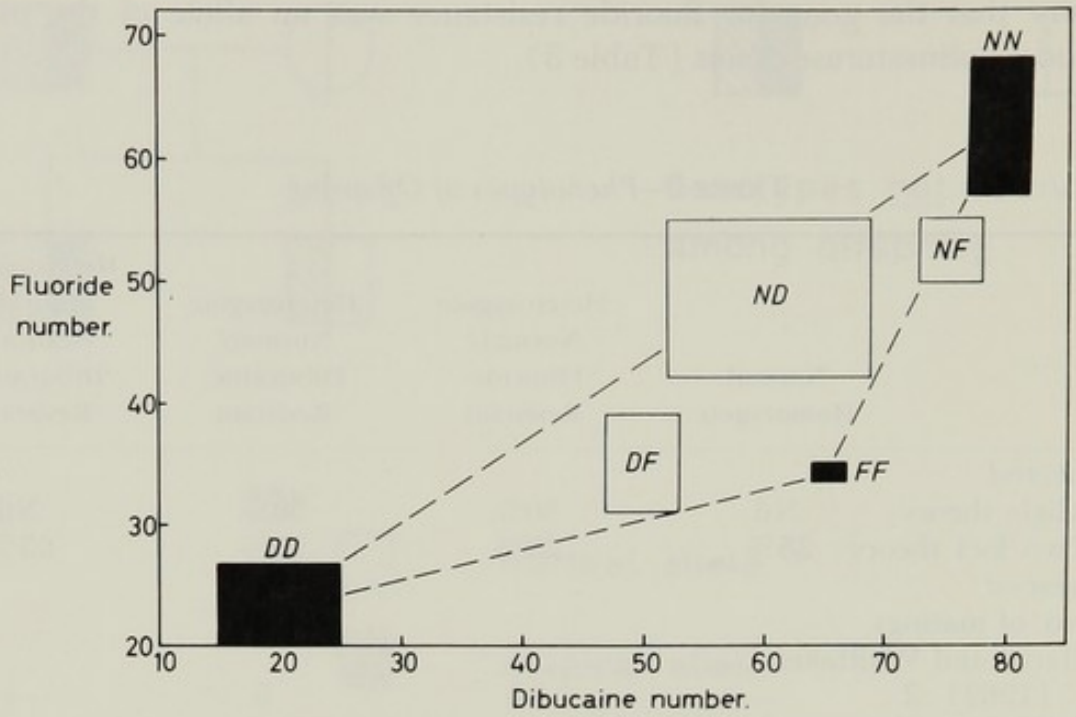


FIG. 10—The relationship between the dibucaine and fluoride numbers of the serum pseudocholinesterase activities determined by the three alleles for: 1) normal pseudocholinesterase (N), 2) atypical or dibucaine resistant enzyme (D), 3) fluoride-resistant enzyme (F). The values found in the three homozygotes lie within the black rectangles, and those of the heterozygotes within the white rectangles. The centers of the rectangles for the heterozygotes should lie on the dotted lines joining the centers for the homozygotes. Since the normal enzyme has a greater activity than the other variants, the values for heterozygotes with the normal and one other pseudocholinesterase gene (*ND, NF*) should lie nearer the normal values (*NN*) than those for the other two homozygotes (*DD, FF*). It can be seen that there is reasonable agreement between theory and observation.

## *The Silent Gene*

It was mentioned earlier that three families have been described in which the dibucaine values do not conform to a simple Mendelian pattern (Kalow and Staron, 1957; Harris, Whittaker, Lehmann and Silk, 1961; Bush, 1961). The details of these families are shown in Figure 11. We have recently seen a fourth such family (Fig. 12) (Liddell, Lehmann and Silk, 1962). In each case individuals appear on dibucaine testing to be abnormal homozygotes, while the values found in their parents predict that they are heterozygous. In addition, in family A (Fig. 11) one such individual ($II_2$) has had an offspring ($III_1$) who appears to be a normal homozygote. This also suggests that $II_2$ must be heterozygous.

A number of explanations were possible. The first is nonpenetrance of the normal gene. The term "penetrance" is really a description rather

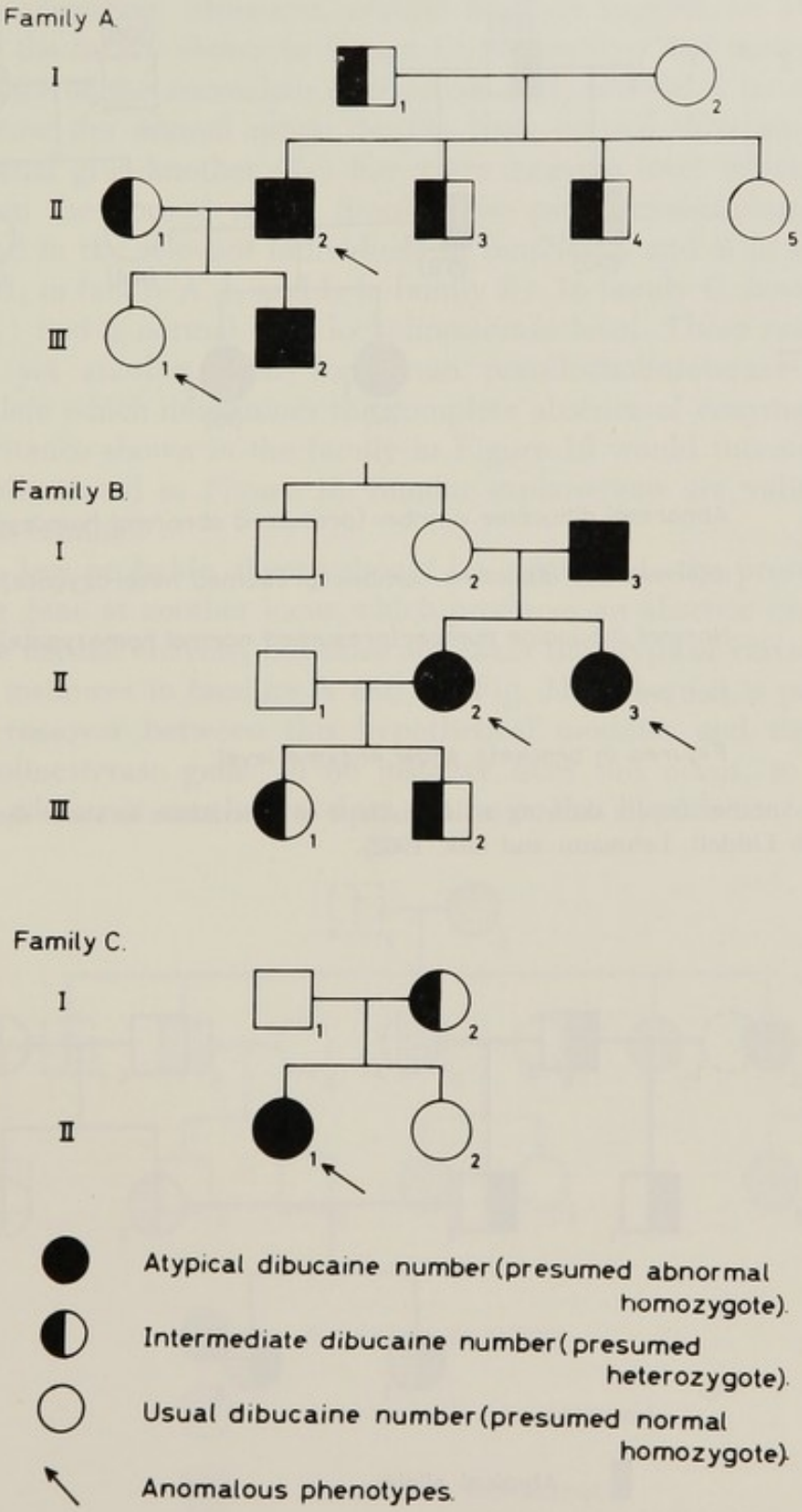


FIG. 11—Three families showing an unusual type of inheritance of the pseudocholinesterase variants. Family A is part of family Y from Kalow and Staron (1957). Family B is from Harris, Whittaker, Lehmann and Silk (1960). Family C is from Bush (1961).

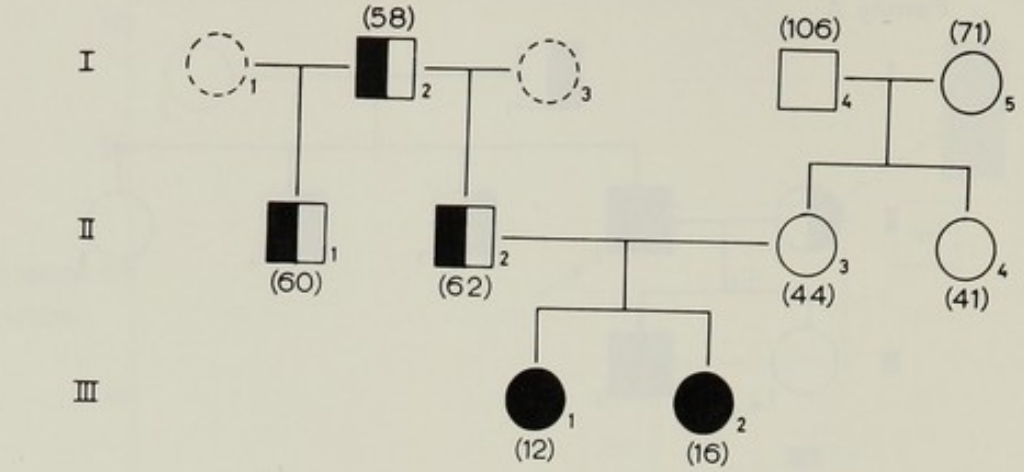


FIG. 12—Another family showing a similar type of inheritance to those shown in Figure 9 (from Liddell, Lehmann and Silk, 1962).

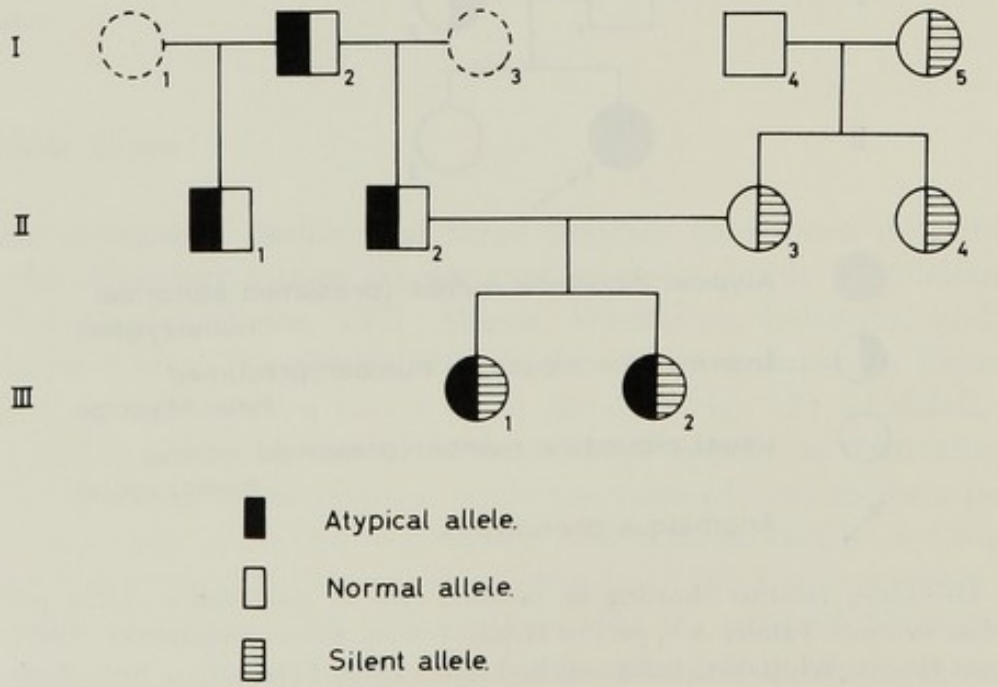


FIG. 13—An explanation of the results shown in Figure 10.

than an explanation. Moreover, certain findings suggest an alternative theory. In the family shown in Figure 12, the mother and maternal aunt ($II_3$ and $II_4$) of the anomalous individuals ($III_1$ and $III_2$) have enzyme values below the normal range despite their normal dibucaine values. The maternal grandmother ($I_5$) has a low enzyme level which is however within the normal range. Similar low pseudocholinesterase levels were found in the relevant individuals in families A and B in Figure 11 ($I_2$ and $III_1$ in family A, $I_1$ and $I_2$ in family B). In family C, however, the father ($I_1$) had a normal pseudocholinesterase level. These results suggest that yet another allele for serum pseudocholinesterase exists, a "silent" allele which determines the complete absence of enzyme activity. The inheritance shown in the family in Figure 12 would therefore be of the type illustrated in Figure 13. Similar explanations are valid for the other three families.

A third, less probable, theory should be mentioned: the presence of a modifying gene at another locus which produces an absence or inactivation of the normal enzyme, but does not affect the atypical variant. There are three instances in families A and B (Fig. 11) where it is possible to test for crossover between this hypothetical modifier and the normal pseudocholinesterase gene. In no instance does this occur, so that the evidence, while not conclusive, does not favor this hypothesis.

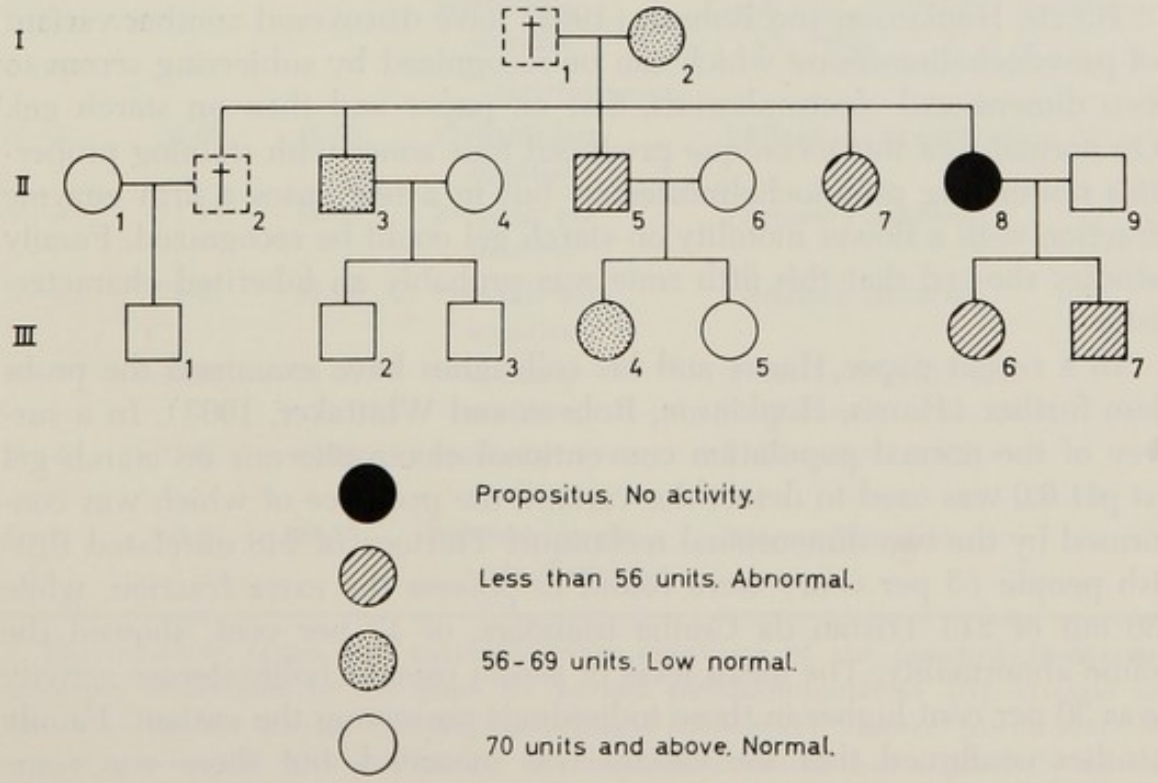


FIG. 14—The pseudocholinesterase levels in the family of a subject with complete absence of enzyme activity (from Liddell, Lehmann and Silk, 1962).

The theory of the silent pseudocholinesterase gene is supported by the existence of an individual with complete absence of serum pseudocholinesterase activity. That this subject is the homozygote for this rare allele is supported by a family study, shown in Figure 14, in which the children and two of the siblings of the propositus, have low pseudocholinesterase levels and are presumably heterozygotes (Liddell, Lehmann and Silk, 1962).

A silent gene may determine either the complete absence of the enzyme molecule or the production of a closely similar protein but with no enzymic activity. Examples of both types of variant have been found in bacterial genetics. It is impossible to decide which is the correct interpretation in the present instance. But it is of interest that the serum of the individual with a complete absence of enzyme activity slightly inhibits the normal pseudocholinesterase. This inhibitor appears to be a protein: it cannot be removed by ultrafiltration, it is thermolabile and it can be precipitated by ammonium sulphate in a manner quantitatively identical with that of the normal serum enzyme. It may be that this inhibitory property is due to the presence of a variant molecule without pseudocholinesterase activity.

*A Variant Detected by Electrophoresis*

Harris, Hopkinson and Robson (1962) have discovered another variant of pseudocholinesterase which can be recognized by subjecting serum to two dimensional electrophoresis, first on paper and then on starch gel. On normal sera this technique produced four zones with staining properties resembling pseudocholinesterase, but in a few cases a fifth enzyme fraction with a slower mobility on starch gel could be recognized. Family studies showed that this fifth zone was probably an inherited characteristic.

In a recent paper Harris and his colleagues have examined the problem further (Harris, Hopkinson, Robson and Whittaker, 1963). In a survey of the normal population conventional electrophoresis on starch gel at pH 6.0 was used to detect the variant, the presence of which was confirmed by the two dimensional technique. Thirteen of 248 unrelated British people (5 per cent) were found to possess the extra fraction, while 36 out of 213 Tristan da Cunha islanders, or 17 per cent, showed the same abnormality. The mean level of serum pseudocholinesterase activity was 30 per cent higher in those individuals possessing the variant. Family studies confirmed that the variant was inherited, but there was some doubt about the mode of inheritance. The most likely seemed to be that the affected individuals were heterozygous for an autosomal gene. But,

in a number of families, neither parent of an affected child showed the trait. This suggests that either the technique is insufficiently sensitive to detect all affected individuals, or that the trait shows a fairly high incidence of non-penetrance. Horsfall and colleagues (1963) did not find this variant in 104 Australian aborigines.

*Suxamethonium Sensitivity*

It now appears that suxamethonium sensitivity can occur in several pseudocholinesterase genotypes. The genotypes are listed in Table 4 and their degree of sensitivity to suxamethonium is indicated.

TABLE 4

| Genotype | Dibucaine No. | Fluoride No. | Suxamethonium Sensitivity | Comments |
|---|---|---|---|---|
| | | | *Homozygotes* | |
| *N—N* | 71-83 | 57-68 | Not sensitive | The normal population |
| *D—D* | 15-25 | 20-25 | Markedly sensitive | Frequency 1:2800 |
| *F—F* | 64, 67 | 34, 35 | Moderately sensitive | One case described (Lehmann et al., 1963) |
| *S—S* | — | — | Markedly sensitive | One case described (Liddell et al., 1962) |
| | | | *Heterozygotes* | |
| *N—D* | 52-69 | 42-55 | Probably not sensitive | 3.8% of the population |
| *N—F* | 71-78 | 50-55 | Probably not sensitive | Frequency unknown |
| *N—S* | 71-83 | 57-68 | Probably not sensitive | Frequency unknown |
| *D—F* | 47-53 | 31-39 | Moderately sensitive | Three cases described (Lehmann et al., 1963) |
| *D—S* | 15-25 | 20-25 | Markedly sensitive | Liddell et al. (1962) |
| *F—S* | ?64-67 | ?34-35 | Probably moderately sensitive | Not yet described |

The inhibitor values and suxamethonium sensitivity of the pseudocholinesterase genotypes determined by the genes for *normal* pseudocholinesteras (N), atypical or *dibucaine-resistant* enzyme (D), *fluoride-resistant enzyme* (F), absence (silent gene) of enzyme activity (S). This is a purely "ad hoc" nomenclature and a generally agreed nomenclature is at present under consideration by all the workers in this field. (See also Chapter 2, Table 2.)

*General Pattern of Molecular Diseases*

The unfolding of the genetic background of pseudocholinesterase is remarkable in the way it resembles that of the hemoglobins. There, a molecular disease—sickle-cell anemia provided the same stimulus as was given to research on pseudocholinesterase by suxamethonium apnea. Investigation of suxamethonium apnea led to the recognition of three genotypes: the atypical homozygote, who is liable to be affected, the heterozygote, and the typical homozygote; just as in sickle-cell anemia there are the abnormal homozygote, the sickle-cell trait carrier, and the normal homozygote. It was discovered that there were modified sickle-cell diseases. The combination of the sickle-cell trait with that for $\beta$-thalassemia, in which formation of normal hemoglobin is impeded, results in a picture very similar to that of homozygous sickle-cell anemia. Similarly, the heterozygous state for the atypical and silent pseudocholinesterase genes, resembles that of the homozygote for atypical pseudocholinesterase. One may compare Sickle-cell hemoglobin C disease with the modified suxamethonium apnea resulting from the combination of atypical and fluoride-resistant pseudocholinesterase, and hemoglobin C disease with the suxamethonium apnea of the homozygote for the fluoride-resistant gene. It thus seems that we can observe the emergence of a general pattern in the inheritance of abnormal proteins and of the pathological states which result.

*Maintenance of Gene Frequency*

The pseudocholinesterase variants differ from other enzyme deficiencies of man, such as glucose 6 phosphate dehydrogenase deficiency and phenylketonuria: it has been shown by Kalow and his collaborators that the most common variant is due not to the complete absence of the enzyme, but to a mutant protein with altered sensitivity to positively charged inhibitors and a slightly reduced electrophoretic mobility. It is tempting to think that this difference is merely apparent, and that many of the other enzyme deficiencies known in man are due to a similar mechanism.

The various environmental factors determining the initial survival and maintenance of this polymorphism are not known. Indeed it is difficult to speculate when even the function of the serum enzyme is uncertain. The health of the individual with complete absence of serum pseudocholinesterase activity indicates that the advantages which the enzyme confers are marginal. Moreover, the close similarities in gene frequencies found in different populations suggests that either the different geno-

types confer little selective advantage or disadvantage under modern conditions, or that the relevant environmental factors are the same in such widely different communities as England and Indonesia.

In only one instance has it been possible to suggest an environmental factor which might be of importance in the maintenance of the polymorphism. Orgell, Vaidya and Dahm (1958) showed that a cholinesterase inhibitor was present in many solinaceous plants such as tomatoes and potatoes. The outer part of the potato contained much higher amounts than the peel. Harris and Whittaker (1959) showed that this inhibitor, like dibucaine, was a differential inhibitor of the pseudocholinesterase variants. At that time it was not realized that Pokrovosky (1956) had shown that the glyco-alkaloid solanine, known to be present in members of the solinaceae, was a cholinesterase inhibitor. Harris and Whittaker (1962b) have now demonstrated that it is indeed this substance which is responsible for the differential inhibition shown by potato extract. Solanidine, obtained from solanine by removal of the carbohydrate residues, shows an even greater differential effect. Since the enzyme found in the abnormal homozygote is less sensitive to this naturally occurring inhibitor, it might be that this genotype would be at a selective advantage in cases of solanine poisoning.

A few outbreaks of solanine poisoning have been reported. In the most recent, four people experienced vomiting, diarrhea and abdominal pain about 8 hours after eating potatoes baked in their jackets (Wilson, 1959). Potatoes from the same batch contained 50 mg. of solanine per 100 Gm. instead of the usual 8 mg. per 100 Gm. The toxic dose is about 25 mg. Other outbreaks involved 61 persons; one of whom died (Harris and Cockburn, 1918) and about 50 cases in Cyprus, again with one fatality (Willimott, 1933).

Koelle, Koelle and Friedenwald (1950) have shown that when intestinal pseudocholinesterase is inhibited the isolated organ shows increased motility. Similar findings have been reported by Burn, Kordik and Mole (1952) who decreased the intestinal pseudocholinesterase content of rats by irradiation. Barnard (1952) has suggested that intestinal pseudocholinesterase destroys cholinesters formed by bacterial action in the bowel, and thus protects true cholinesterase against the inhibitory effect of these compounds. It is therefore of interest that the symptoms of solanine poisoning include vomiting and diarrhea developing several hours after ingestion. This suggests that at least part of the symptoms are due to the inhibition of intestinal pseudocholinesterase.

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Chapter 4

# Transplantation Antigens in Mouse, Rat, and Man

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## *Introduction*

The analysis of transplantation or histocompatibility antigens in man is complicated by the existence of very many antigenic factors, and the difficulty of assessing whether an individual antigen is important or inconsequential. Before large scale investigations into the tissue antigens of man are begun, it seems logical to see what can be learned from previous experience in lower animals. This article will discuss certain aspects of knowledge of transplantation antigens in mice and rats, will discuss some of the better documented antigens in man, and will then examine some of the criteria for establishing the existence of new factors. This will not be an exhaustive survey of the literature, since many good reviews and extensive bibliographies are available, but will be restricted to a consideration of some special features of transplantation systems.

The terms "transplantation antigen" and "histocompatibility factor" are generally used interchangeably to denote substances on or near the surface of a cell which induce immunity in a recipient animal of different antigenic constitution but which are not antigenic for a genetically similar host.

Many antigens are present on both red cells and leukocytes and there is a close similarity in the immunity to either. While the study of red cell

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antigens has progressed rapidly, the identification and characterization of transplantation antigens has lagged behind. Efforts in experimental transplantation have been concerned mainly with studies on immunological mechanisms.

There are obvious reasons for this difference in emphasis. Red cell antigens have been amenable to study because many of the antibodies directed against them are readily demonstrated and extremely sensitive techniques have been devised for the recognition of weak reactions. Once the ABO groups could be matched, blood transfusion became a practical therapeutic routine and innumerable transfusions have been performed.

Following the recognition of the major red cell antigens additional factors were identified by analyzing sera of patients with transfusion reactions. (Only relatively recently has it been found that many of these reactions are against white cells.) Many of these factors have little practical importance in transfusion but have been of the greatest value in genetic studies; thus in plotting the prehistoric migration and distribution of peoples, or the evolutionary relationships between man and other species the study of blood group gene frequencies has been widely used. Studies of relationships between blood group genes and susceptibility to disease, and the use of blood group genes in linkage studies are progressing rapidly. At the subcellular level, knowledge of the mechanisms of genetic control and of the elaboration of blood group antigens is of great theoretical value. The study of alterations in the amount of these antigens in leukemia and gastric cancer and of antibodies to red cell antigens in auto-immune diseases are two more examples of the way in which information concerning these antigens is being exploited. This is not the case with tissue antigens where no simple antigenic factors comparable in relative importance to the ABO groups have been found, and where matching for compatibility is at present not possible.

There is, however, no fundamental difference between the iso-antigens of red cells and those of the white cells and fixed tissues. The immune response to red cells is thought to be predominantly humoral, while that to tissues to be due to lymphocytic reactions. This is not a true distinction. Destruction of incompatible human red cells appears to be extravascular except in very severe cases of ABO incompatibility, while antibodies can generally be detected after graft destruction.

It is necessary to explain the reason for re-emphasizing this rather obvious point. Although heterospecific antibodies to various cell types including cancer cells, white cells and sperm were demonstrated soon after antibodies to red cells were known, the recognition of iso-antibodies following transplantation had to await proper experimental methods. Iso-antibodies were often more difficult to demonstrate than heterospecific

antibodies, and while antibodies that cross reacted with red cells could be found, demonstration that these were the antibodies involved in graft rejection was impracticable. Recognition that iso-antibodies were effective in destroying tumor cells came largely through the work of Lumsden (1937 and 1938), followed by Gorer and his colleagues (Gorer, 1942; Gorer and Amos, 1956; Gorer and O'Gorman, 1956) and more recently Stetson and Jensen (1960) and others (Chutna, 1959 and 1961; Steinmuller, 1962) have shown similar effects with skin grafts. This work has taken nearly 30 years; in the meantime, because antibody seemed so ineffective, the attention of many investigators was caught by the potentials of lymphoid cells. Transferred lymph node cells were found by Medawar and his colleagues to be effective *in vivo* (Billingham, Brent and Medawar, 1954; Mitchison, 1955), hence many studies have exploited this manifestation of immunity (see Brent, 1955). *In vitro* demonstration of cellular activity was, however, as slow in coming as the *in vivo* demonstration of antibody effect. Finally, after repeated failures, success has been reported from many laboratories (Koprowski and Fernandes, 1962; Rose et al., 1963; Rosenau and Moon, 1961; and see cell-bound antibodies). The present concept of mechanism of graft rejection may be summarized as follows. Following immunization with cellular material, especially with live cells, an allogeneic (Boyse, Old and Stockert, 1962; Slettenmark and Klein, 1962; Steinmuller, 1962), or a heterospecific* host (Haskoya, Chutna, Hasek and Hort, 1962) will develop antibodies which are cytolytic and can destroy the graft. The antibodies may conveniently be measured by properties such as agglutination (Amos, 1953; Gorer and Mikulska, 1954), cytolysis (Winn, 1962) or complement fixation (Batchelor, 1960). Passive immunity can be transferred by antibody under suitable conditions (Amos and Day, 1957; Gorer and Amos, 1956). The lymphocytes and macrophages of the host also show changes referable to the immunization and can also transfer immunity (Amos, 1961; Baker, Weiser, Jutila, Evans and Blandau, 1962). These changes cannot be dissociated from those due to antibody: the reactive

* Terms used: Autologous—pertaining to the individual; syngeneic—a different individual of identical genotype; allogeneic—of different genotype but within the same species; heterospecific—of different species; iso-antigen—a substance recognized as antigenic by genetically dissimilar host of the same species; homograft—a graft within members of the same species, qualified by syngeneic or allogeneic depending upon whether donor and host are similar or of different antigenic types, respectively; heterograft or heterospecific graft—a graft between members of different species.

Examples: A skin graft of C57BL skin to a C57BL mouse is a syngeneic homograft, and is accepted. A similar graft to a C3H mouse is an allogeneic homograft and is rejected with the formation of iso-antibodies which react with iso-antigens present on C57 cells. The same graft on a rat is a heterograft and the antibodies formed are predominantly heterospecific or species specific antibodies.

specificity of immune cells may be less rigid than that of antibody, but at least part of the activity of the immune cells seems to be due to special kinds of antibody which may be selectively adsorbed onto the reactive cells or formed by them (Boyden, 1963; Boyden, Sorkin and Spärck, 1960). Circulating antibody may act synergistically or antagonistically with the immune cells or the two may function independently (Batchelor, Boyse and Gorer, 1960; Batchelor and Silverman, 1962; Gorer, 1960). The reader particularly interested in this topic is referred to articles by Medawar, 1959; Gorer, 1961; Brent, 1955; Amos, 1962; and to a number of excellent symposia (see Biological Problems of Grafting; Cell-bound Antibodies; Cellular and Humoral Aspects of the Hypersensitive States; Ciba Foundation Symposium on Cellular Aspects of Immunity; Ciba Foundation Symposium on Transplantation; Fifth Tissue Homotransplantation Conference; Proceedings of the International Symposium on Tissue Transplantation; and Tumor Immunity.)

Red cells and other tissues, then, share many antigens both species and individual specific. Some red cell antigens appear to be confined exclusively to the red cell but the search has not been comprehensive. Sometimes the data are scanty and there remains controversy over the distribution of such well studied antigens as those of the Rh system. There are often marked quantitative differences not only between the amount of antigen on red cells and other tissues, but also between the various organs and tissues of the body. These differences could be expected from a consideration of the specialization of function of most tissues and therefore to the consequences of differential gene action.

Antigens vary greatly in their reactive strength (Berrian and Jacobs, 1959) and in the time of their appearance in the embryo (Möller, 1963; Möller and Möller, 1962). These factors are of consequence in the establishment of tolerance and in the etiology of autoimmunity. Late development of an antigen is of little moment if the substance does not reach the surface of a cell exposed directly or indirectly to vascular channels. Various substances in brain, testes and thyroid are well documented examples. If, however, the antigen appears on the cell surface after the honeymoon of tolerance is over, immunity occurs. Direct proof of this has been offered in an ingenious experiment in which the buccal component of the pituitary of the tree frog was removed soon after birth, maintained in culture, and replaced after immunologic maturity had been reached. There was a typical first set reaction to the implant which was not seen following implantation of gland into partially hypophysectomized animals (Triplett, 1962).

Antigens in contact with the vasculature appear to be present at birth or to appear in quantity shortly after birth. One of the objectives of

studies on tissue transplantation is to induce the individual to revert to a state of specific immunological tolerance with respect to the tissue antigens of a putative donor. It appears that this can best be achieved by first selecting a donor with as many relevant antigens in common with the recipient as possible, and by then attempting to modify the host, possibly following the model proposed by Uphoff (Uphoff, 1958 and 1961) of giving a suitable immunologic depressant such as Amethopterin together with a fairly large quantity of antigen. The physical state and route of administration of the antigen may be as important as the choice of depressant (Medawar, 1963), while biological products such as $\beta$-lipoprotein may be found to be safer and more suitable for use as depressants (Kamrin, 1960; Mowbray, 1963) but these are outside the scope of this article which will discuss the genetic factors in transplantation immunity.

## Transplantation Antigens in the Mouse

### *H-2*

The H-2 system appears to be unique in its complexity and in the degree of reactivity it provokes in an allogeneic host. H-2 incompatibility is not readily overcome; skin or tumor graft rejection between mice incompatible for H-2 is usually rapid and tolerance is established even in newborn animals only with difficulty, depending upon the strain combination and nature of the tissue used (Billingham and Brent, 1959; Billingham and Silvers, 1962; Russell, 1962). Injection of spleen or lymph node cells into allogeneic newborns frequently causes runting and death (Billingham and Brent, 1957 and 1959; Billingham and Silvers, 1961; Russell, 1962). The antibody response to H-2 is readily detected and the H-2 antibodies have potent cytotoxic and protective activities against transplanted tumors as well as having strong hemagglutinating properties (Gorer and Amos, 1956; Gorer and Mikulska, 1959). In addition, the H-2 antigens appear to be the last to be suppressed or lost from a transplanted tumor during the antigenic simplification known as tumor progression (Hauschka and Amos, 1957).

H-2 was first referred to as antigen II by Gorer (1937). It was soon found by Gorer, Lyman and Snell that there was close linkage between the loci controlling H-2 and "Fused," a tail abnormality on the IXth linkage group (Gorer, Lyman and Snell, 1948). The presence of the H-2 antigen could be recognized in a suitable outcross to a fused stock. One of the crosses used was between A and CA, a stock heterozygous for fused. The resultant $F_1$ was outcrossed to mice with a different H-2

"allele." The mice were then examined for the phenotypic expression of fused, for the agglutinability of their cells by an antibody against A, and for their ability to accept an A strain tumor. Close linkage was found between the three characters; fused mice did not accept tumors and their red cells were not agglutinated; mice with normal tails were susceptible and carried the agglutinogen. There were some discrepancies in the expression of the characters in the cross to A which could be explained as lack of penetrance or as cross overs between fused and H-2. These discordances were not observed in another cross involving DBA (Gorer, Lyman and Snell, 1948).

Even at this time it was recognized that there were several "alleles" of H-2, but the extreme complexity of the H-2 system was not recognized until better methods of serological determination were developed by Gorer and Mikulska (1954). Serological studies were pursued by Gorer and his colleagues (Amos, Gorer and Mikulska, 1955; Gorer and Mikulska, 1959; Hoecker, Counce and Smith, 1954), tumor rejection in outcrosses by Snell et al. (Snell, 1952 and 1957; Snell, Smith and Gabrielson, 1953). Tumor transplantation indicated that there were numerous forms of H-2 and these were regarded as "alleles" by Snell (1951). In the recombination studies, as with the agglutination and absorption analyses, there was considerable cross reactivity between certain strains, and Gorer came to believe that H-2 was a system of many closely linked genes, each existing in two or more allelic forms (Amos, Gorer and Mikulska, 1955). Because this did not fit the usual concept of gene or locus, he preferred to refer to the H-2 'region.'

A number of antisera was prepared to cover most of the possible combinations within 4 inbred strains, A, BALB/c, C3H, and C57. The cross reactions before and after absorption with liver from other strains were analyzed and certain factors peculiar to the strain or shared with other strains were recognized (Amos, Gorer and Mikulska, 1955).

Serological studies in which new strains of mice have been included have expanded the list of recognizable factors. A number of factors have never been recorded in print because of problems of nomenclature, and it is probable that over 25 distinct reactivities have been observed (Amos, 1959 and 1962; Hoecker, Counce and Smith, 1954; Hoecker, Pizarro and Ramos, 1959; Stimpfling and Snell, 1962).

The genetic control of H-2 appears to be by a series of subdeterminants, each controlling one antigen, which generally act synchronously to give a uniform pattern of antigenicity for the cells of any given genotype. Variations between tissues appear to affect all the components equally. Spleens from two strains, for example, have the same relative amount of antigen regardless of H-2 genotype while kidneys from the same strains have very

little. While the genes tend to act in concert and are activated at about the same time during development, the genes and gene products appear to have a high degree of individual specificity and to behave as discrete units. Variations in the amount of antigen on the red cell have been noted; for example, the antigen E is present in only small amounts on the red cells of C57BL mice although other tissues such as liver or spleen carry abundant amounts of this antigen. This appears to be a characteristic of the genotype, since strain 129 and the now extinct Pearl strain, neither of which appeared to have common ancestry with C57BL, have this peculiarity. F/St cells appear to have an unusually large amount of antigens E, F, and A. Variations in the amount of antigen are often found in aneuploid tumors where a given antigen may be present in greater or lesser amount than in the corresponding parental tissue (Amos, 1962, and unpublished data). An example of loss of antigen K or K and D but not D alone was thought to be an example of somatic crossing over although the data could equally be interpreted on the basis of deletion (Hellstrom, 1961; Klein, 1961; and see Amos, 1962).

Recombination within the H-2 region was independently reported by Allen (1955) and by Gorer and his colleagues (Amos, Gorer and Mikulska, 1955) and has since been observed on a number of occasions. A linear position of genes has been proposed, D and K appear to be near the ends of the region and are separated by a segment of chromosome which carries the genes controlling the antigens C, V, M, and H. D and F have not yet been separated in the recombinants, neither have K and E. D and K appear to be at most 1.5 cross over units apart (Pizarro, Hoecker, Rubinstein and Ramos, 1962).

Three of the recombinants have been studied in some detail (Gorer and Mikulska, 1959) and the progeny have been inbred for several years. Five further examples are under investigation by Stimpfling (personal communication); four of these have been described in a preliminary report by Stimpfling and Snell (1962).

Complete analysis of H-2 has been hindered by the lack of serological reactivity of some of the allelic forms of many of the factors. Just as the antigens of C57BL red cells cannot be demonstrated as agglutinogens without the use of developing agents such as long chain polymers, so some of the other antigens cannot be detected at all readily. For example, the antibody A anti C3H should react with the alleles of D, F and M. While the antibody agglutinates C3H white cells, it generally fails to agglutinate C3H red cells, although the existence of the alleles can be inferred by the ability of an A mouse to reject a C3H graft. Fortunately, it has recently been found that the antibody reacts vigorously with C58 red cells and it should be possible to see whether the allele $D^k$ of D is also present

on other strains such as C57. The analysis of the H-2 antigens of some of the common strains is still not completed and offers some interesting problems.

Although recombination has been observed on many occasions within H-2, mutation is poorly documented. Several examples have been recorded in the literature but in each case there was a strong possibility of contamination since the animals were obtained from a commercial production source and not from a closely pedigreed stock.

It is obvious from the complexity of the known H-2 genotypes that mutation as well as recombination must occur. Point mutations have not yet been documented, and might be missed unless they involved one of the better recognized factors. It is possible that the I, N, and JK stocks derived by Strong from a pen-bred stock differ from each other by one or two point mutations and that the C3H subline of Figge differs only from the H-2k of C3H in carrying the gene for antigen F, but it has not been proved that single antigens only are involved.

Polygenic mutations may have occurred in a polydactylous subline maintained by Strong (1962) but other changes were also present which can best be explained by the effects of a mutator gene. The "new" phenotype differs by two or more antigens from the parent strain.

It is notable that very few of the possible H-2 combinations have ever been described. A few particular combinations have been found repeatedly, thus there are "favored" genotypes in mice, and this is a rich field for future population studies (Table 1).

Certain combinations such as H-2k, H-2d, H-2a and H-2b have been repeatedly encountered in unrelated stocks. These 4 combinations are the most frequently found genotypes, but in addition "groups" occur. The H-2 antigens of C58 and RIII resemble those of the H-2k type strains, but are not identical, YBR is very like the H-2d group, and the series of lines derived from Bussey Institute mice by Strong (1942), I, JK, N and PBR form a group with generally similar antigenicity although with certain marked individual differences.

It was thought at one time that the antigen was dispersed through the cell and later that it was mainly associated with the nucleus. Three different methods for determining immunity have been employed, and three different corresponding H-2 "antigens," enhancing, transplantation, and histocompatibility, were postulated to account for the apparent discrepancies. This is no longer thought to be correct. The general concept of immunity to H-2 is that the individual reactions are determined by specific groupings bound to a common complex skeleton. Haughton (1962) and Davies (1961) believe that there is a protein backbone which may be identical with an antigen which they have identified as antigen III, while

TABLE 1—*H-2 Genotypes and Some Characteristic H-2 Antigens*[1]

| H-2 Genotype | Strain | | Some Characteristic Antigens |
|---|---|---|---|
| | With characteristic H-2 genotype | In same general group | |
| a | A, AKR.K | L/St, SWR | A,C,D,E,F,H,J,K,M,N,Y,A′,B′,C′,R. |
| b | C57BL, C57L, 129 D1.LP, B10.BY, B10.LP, STA, C3H.SW | Poly A/St | $D^b$,E,F,V,N,A′,B′,C′,R. |
| c | D1.C | — | C,D,E,H,M,A′,B′,C′. |
| d | DBA/2, BALB/c, C57BL/Ks, B10.D2, C57BL.H-2d, ST.T6, WH | YBL,YBR | C,D,$E^d$,F,H,J,M,N,A′,B′,C′,R. |
| e | STOLI | — | C,E,F,M,Y,R. |
| f | A.CA, B10.M | — | C,G,H,I. |
| g | H-2G | — | $D^b$,$E^d$,F,N,V. |
| h | H-2H | — | A,C,$D^b$,E,F,H,K. |
| i | H-2I | — | C,D,E,F,M,V. |
| j | JK | — | F,V,G? |
| k | $C3H^2$,CBA,CHI,CE,ST, 101,MA,$RF^3$,STB,D1.ST, AKR,AKR.ALB,C3H.K | C57Br/A?, C58, C57Br/cd? | A,C,E,H,K,Y. |
| l | N | I,PBR,PRUNT,Poly B/St | F,J,V. |
| m | AKR.M | — | C,E,H,K,A′,B′,C′,D′. |
| n | F | — | A,E,F,H,J,N,R. |
| p | P,$C_3$H.NB | BDP? | C,E,F,P. |
| q | DBA/1,C/St, BUB, B10.Y | CF | C,E,F,K,M,Q,A′,B′,C′,D′,R. |
| r | RIII | LP.RIII | C,E,H,K,R,Y. |
| s | A.Sw | S.JL | C,E,F,G,R,S,B′. |

[1] Compiled from (Amos, 1962; Gorer and Mikulska, 1959; Hoecker, Counce and Smith, 1954; Hoecker and Pizarro, 1962; Hoecker, Pizarro and Ramos, 1959; Stimpfling and Snell, 1962 and Amos, unpublished observations).

[2] $C_3$H/Rij appear to have antigen F but not K.

[3] RF carried at Bar Harbor appear to me H-2k; RF carried by Upton are not. This variant is being examined by Popp (personal communication).

Castermans (1961) has speculated that the type of response might be determined by a "carrier." There is a strong possibility that the basic molecule is not the same in all tissues although the antigenic specificity is. The lack of complete correlation between the level of circulating antibody and the clinical demonstration of transplantation immunity reflects the over-all result of at least three factors; humoral, cellular, and vascular, which participate to varying degrees depending upon the nature of the antigenic stimulus, the site of the graft and the genetic diversity between graft and host. Different forms or other chemical groups associated with the antigen molecule could greatly modify the effective balance of these factors.

By antibody inhibition tests it was possible for Basch and Stetson to show that the greatest concentration of antigen was present in the lysosomal fraction of a cell hemogenate (Basch and Stetson, 1962), but at least some antigen is known to be present on the cell membrane. Kaliss and his colleagues (summarized by Kaliss 1962) had shown earlier that antigenic activity could be found in most cell fractions including the supernatant after extraction with butanol.

*Chemistry of the H-2 Antigens*

Partial purification of the H-2 antigen has been attained by a number of investigators. The most active end product appears to be a water insoluble lipoprotein containing very little carbohydrate or sialic acid and up to 40 per cent lipid (Davies, 1962). There are certain differences between the products obtained by different investigators, probably due to differences in the physical state of the complexes obtained. Davies and Hutchison have reported on a preparation which when tested *in vitro* inhibits hemaggultination at the low level of 8$\mu$g./ml. (Davies and Hutchison, 1961), and similar activity is reported for a preparation obtained by Manson by a modification of the method previously reported (Manson, Foschi, Duplan and Zaalberg, 1962), and tested by Palm (Manson and Palm, 1963). Herzenberg and Herzenberg (1961) have a product generally similar to that of Davies (1962), while Kandutsch (Kandutsch, 1960; Kandutsch and Reinert-Wenck, 1957) has a triton soluble antigen which becomes water soluble after treatment with snake venom. Preparations made by Davies, Kandutsch and Herzenberg were compared and all were found to be active *in vitro* and to stimulate hemagglutinin production *in vivo* (Amos, Haughton and Spencer, 1963; Kandutsch and Stimpfling, 1962). A preparation of similar chemical constitution but obtained in a radically different manner and with dissimilar physical characteristics with respect to solubility and stability, has been reported by

Castermans and his colleagues (Castermans and Oth, 1959; Lejeune and colleagues, 1962). Unfortunately, although both high antigenic activity *in vitro* and sensitization *in vivo* have been reported, the only sample available for study in our laboratory was inactive.

Brent, Medawar and Ruszkiewicz have studied the properties of water lysates (Brent, Medawar and Ruszkiewicz, 1961) and found that the ability to induce immunity against subsequent skin grafts was still present after exposure to pH 11.0 or after heating to 100° C for 4 minutes. These authors found that some of their preparations would still immunize but could no longer absorb H-2 hemagglutinins, and speculated that the sensitizing action could have been due to antigens of other systems (Brent, Medawar and Ruszkiewicz, 1961). Brent et al. (1962) and Lejeune et al. (1962) found that the lipid insoluble fraction of a cell homogenate retains its activity after extraction, whereas Davies found that his more purified material loses activity as the lipid content is reduced. The major differences between the stability to extremes of pH and to enzymic action could be explained if some of the antigens other than H-2 are chemically different.

The measurement of immunological activity of an antigenic preparation can be measured *in vitro* by its ability to absorb antibody and *in vivo* by its ability to produce an alteration in the immune status of the host, usually by formation of antibodies or by manifestations of immunity, tolerance or enhancement. A discrepancy does not necessarily indicate a difference in antigenicity. Different products are handled differently by the animal, thus Medawar found that the same preparation could be ineffective by intravenous route but highly antigenic intracutaneously. This has been well documented for soluble antigens by Leskowitz and Waksman (1960), where again intravenous administration was relatively ineffective in stimulating either delayed hypersensitivity or antibody production whilst intradermal inoculation was effective for both. Interestingly, these differences disappeared after hyperimmunization.

A substance may be highly antigenic *in vitro* and inert in *vivo* if it is eliminated without stimulating the lymphoid system or if it represents a hapten rather than a complete antigen. The same preparation may become highly antigenic *in vivo* if its form is only slightly modified, or if it is conjugated. Several investigators have noticed that liver, while being an effective antigen as judged by its ability to absorb antibody *in vitro*, is very inert in stimulating antibody formation *in vivo*. Spleen is an effective antigen in both respects. Hoecker and Pizarro (1962) and Castermans (1960) believe that there are two distinct antigens. Hoecker has quoted data of Pizarro and Moreno indicating that spleen has a heavy antigen found also in several other tissues and another remaining in suspension at

11,000 g which is not found in supernatants of lymph nodes, thymus or other tissues. Castermans (1960) has separated two distinct activities by electrophoresis at pH 7.5, of three different preparations of spleen and thymus. One active material remained at the origin; the other migrated for 15-23 cm. towards the cathode. Kaliss et al. (1963) believe that enhancing antibodies are distinct from the H-2 hemagglutinins. These repeated indications of different forms of antigen or antibody are reminiscent of the duality of the human blood group A substance, a water soluble antigen of low molecular weight and an alcohol soluble antigen of more complex structure, both with similar antigenic specificity but with very different immunochemical reactivities.

This area is one of intense activity, and great caution is needed in interpreting results, especially where apparently contradictory and conflicting results are obtained. The "antigen" obtained by most investigators is probably a fragment of a larger structure, greatly distorted in its molecular configuration by the extraction methods used.

Even the purest of the H-2 preparations is probably still grossly impure. The only objective measurement of purity is to measure the concentration of a different antigen in the starting material and then in the product. The product of Davies obtained from the C3H tumor BP8 is effective in very low concentration. BP8 is itself a rich source of H-2 antigens but is deficient in many other antigens known to be present in C3H mice. One antigen has, however, been detected (tentatively named antigen 12). The proportion of antigen 12 to H-2 in the purified H-2 product appeared to be similar to the proportion in the native tumor (Amos, Haughton and Spencer, 1963). It should be repeated that nothing is known of the chemical or physical properties of the many other transplantation antigens of the mouse.

## Other H Systems of the Mouse

### *H-Y*

It has long been known that transplantation was most successful if graft and recipient were of the same sex, but it remained for Eichwald and Silmser to show that male skin was promptly rejected by females of the same inbred strain (Eichwald and Silmser, 1955). Hauschka (1955) explained the result by postulating a Y-linked antigen and numerous laboratories have confirmed the hypothesis. Hauschka et al. later suggested that control of the antigen may be from Y heterochromatin and not from a specific gene (Hauschka and colleagues, 1959).

Interest in this antigen centers around the observations of Zaalberg (1959) and of Billingham and Silvers (1960) that all males regardless of the strain of origin have the same Y-linked antigen, H-Y, and of Hauschka and numerous others that the females of certain strains are unable to reject male skin (see Hauschka and colleagues, 1959). The antigenic difference between male and female also provides a very simple isogenic system readily available in every laboratory.

Three hypotheses have been advanced to explain the variable rejection rate between females of different strains. Michie and McLaren believe that certain females are immunologically incompetent to react against the relatively weak stimulus of H-Y (Michie and McLaren, 1958). Klein and Linder have reinforced this and postulated two dominant antigens, either of which must be present before rejection is accomplished (Klein and Linder, 1961). Hauschka and his colleagues have reasoned that there is translocation of part of the Y heterochromatin, that a small fragment of the Y becomes attached to an autosome, and that females carrying this fragment of Y chromosome produce a certain amount of H-Y and therefore cannot react against it (Hauschka, Meagher and Holdridge, 1961).

In support of the weak reaction hypothesis is cited the reactivity of certain $F_1$ hybrids between a nonreactive female and a male of a reactive strain (Zaalberg, 1959), a finding which has been expanded by Klein and Linder (1961) based on their studies of the spread of survival times of skin grafts in a backcross to suggest that the ability to reject depends upon the presence of one of two genes for resistance. Thus if either gene for resistance is present in the female, the presence of the Y antigen will trigger an immune reaction.

The fact that almost every strain of mice shows a proportion of females capable of rejection would imply that even the most strictly inbred lines of mice are heterozygous for one of the "resistance" genes—a suggestion likely to be regarded with scepticism by many geneticists. More serious criticism is provided by Hauschka's reevaluation of the data (Hauschka and Holdridge, 1962). Many of the $F_1$ combinations between accepting females and C57 males do show a high proportion of graft acceptance and there are radical differences between the results obtained with essentially similar mice. Thus Zaalberg strengthened his argument by showing that only 13 per cent of $F_1$ females (CBA x C57BL) accepted C57 male skin, while Hauschka found 82 per cent acceptance of C57BL male skin by reciprocal $F_1$'s between C57 and C3H.

Hauschka has also found that in one subline of Y/Heston mice certain female mice will reject grafts of female skin, and has then shown that female rejectors of female skin are those females which can reject male grafts. Female acceptors of male skin will accept female skin. Thus,

according to his hypothesis, the acceptor females themselves carry H-Y and elicit an immune response in the "normal," male-rejecting females (Hauschka and colleagues, 1959; Hauschka and Holdridge, 1962).

Attempting to confirm the presence of Y antigen in the female, Billingham and Silvers set out to induce tolerance in newborn C57BL mice with bone marrow from mice from another line which were known to be male compatible. Tolerance was induced by male but not by female marrow (Billingham and Silvers, 1960). Although these investigators regarded their data as final evidence against translocation, it has been criticized by Hauschka because quantitative aspects of tolerance were ignored. If the female cells carry less antigen than male cells, then tolerance would require many more female cells for its induction; no quantitative measurements were made in the single experiment performed.

This is an extremely provocative question. Further evidence will undoubtedly come from crosses involving male-compatible females, and through the further use of tolerance or the second set reaction to demonstrate identity between two hypothetical sources of the male antigen. Serological evidence would be of the greatest value. The only reactions described to date were obtained with sera from C57BL females which had rejected C57BL male grafts (Hauschka and colleagues, 1959). Although both cytotoxic and agglutination reactions were observed the reactions were too weak to have any practical value. Attempts to produce a more reactive serum by hyperimmunization have been unsuccessful. Attempts to demonstrate anti-Y against mouse red cells (Sachs and Heller, 1958) were also unsuccessful.

### *X Antigen*

Barrett, Hansen and Derringer (1956) showed a rather indefinite histocompatibility effect which could have been associated with an X chromosome from a grandparent, and subsequently Uphoff (1963) has shown a possible maternal cytoplasmic inheritance in a C3H subline implanted as embryos into C57 females. An X-linked influence on the acceptance of the DBA/2 lymphoma has also been reported by Hauschka et al. (1956); Popp (1961) has discussed the effect of X-linked antigens in the rejection of transplanted bone marrow, but it remained for Bailey (1963) to give a clear demonstration of an X-linked antigen in certain combinations. The discovery of X linkage, like the earlier detection of Y linkage, was fortuitous and arose from experiments to induce mutations at histocompatibility loci by X-irradiation. Reciprocal hybrid BALB/c (C) x C57BL (B) male grafts were put onto similar reciprocal hybrid males. BC hosts accepted 342/344 BC male grafts, but CB hosts rejected 34/34 grafts from the same

BC donors. CB grafts were accepted by 90/92 (CB)$F_1$ males, but only by 22/36 (BC)$F_1$ males. Corresponding grafts of female skin to female hosts showed almost complete acceptance. As in the case of the Y-linked antigen, there are some slight anomalies, but the main observation seems well documented, and a marked second set effect is observed.

*Other Iso-Antigens*

1) *Genetically Characterized Factors.* The existence of many H antigens is apparent from an examination of the proportion of the $F_2$ between two inbred strains which will accept a skin or ovarian graft from one of the original parent lines. For any two unrelated lines, an estimate of as many as 14 genes is obtained. Since many factors are probably common to any two strains, the total number of factors segregating is probably very high. Some 10 factors have so far been tentatively identified, but published data are available for only three of them, H-1, H-3 and H-4 (Counce and colleagues, 1956; Snell, 1959; Snell and Stevens, 1961; Snell, Wheeler and Aaron, 1957). Descriptions of H-5 and H-6 are in press (Amos, Zumpft and Armstrong, 1963).

The most fertile means of characterization of unknown antigens has been that of developing co-isogenic lines as practiced by Snell (1958). Suppose strain A has a character to be investigated; strain B does not have the character. The $F_1$ between A and B is backcrossed to B and the progeny mater *inter se*. These progeny, containing mice of the genotype AA, AB and BB, are challenged with a B tumor and only the homozygous AA survive. These are then crossed with B and the whole process repeated.

Serological methods are now being used to accomplish the same objective provided an anti-A is available. The serological method has the immense advantage that the heterozygote can be identified and mated directly to a BB mouse, thus avoiding many unnecessary $F_2$ generations. It has the disadvantage that there is uusally no known linkage of the H gene to any distinctive marker.

H-1, H-3 and H-4 are found on a number of tissues. Spleen and embryo tissue have been used extensively to immunize and give protection against subsequent challenge with tumor. All are relatively weak antigens and exist in a number of allelic forms, some of which remain to be identified. H-1 and H-4 are both found on the first linkage group. H-1 was detected because of its linkage to albinism and H-4 through its linkage to pink eyed dilute (Snell and Stevens, 1961). H-3 on the 5th linkage group is linked with the gene for agouti (Snell, 1958). There is no evidence that

any of these factors is a compound locus, and H-4 has been found only in two allelelic forms.

H-1 and H-3 have been used in a number of transplantations studies of bone marrow, following lethal irradiation (Uphoff and Law, 1959), and of skin (Berrian and McKhann, 1960; Counce et al., 1956). Tolerance is quite readily effected across an H-1 or H-3 barrier. Mice of the same H-2 genotype do not suffer from the lethal disease commonly found after protection with bone marrow (Uphoff, 1960, 1963). H-1, H-3, H-4, H-Y, and "H-X" are all relatively weak antigens.

H-5 and H-6 have been detected serologically. The antigen H-5.A is present on C3H/St and H-6.A on C3H/He tissues and tumors. Antibodies to both of these factors act as hemagglutinins and there are considerable quantities of both antigens, especially of H-5.A, on the red cell. Both appear to be single factors since cross absorption with tissues from any of the several strains carrying the factor removes all agglutinating activity and both give a single factor ratio in a backcross or $F_2$. Their potency as transplantation antigens is not known but is probably low since neither is strongly cytotoxic. The tissue distribution of both is known, and has been found to differ radically from that of H-2 (Amos, Zumpft and Armstrong, 1963).

A study of the organ distribution of transplantation antigens is likely to have important implications for organ transplantation. Evidence is already available that transplantation of ovary, for example, involves fewer antigens than those effective in skin graft rejection and possibly some different ones (Barnes and Krohn, 1957; Hicken and Krohn, 1960; Krohn, 1959). There also appears to be a greater resistance to the effects of transplantation immunity. Linder found that when ovaries were placed in $F_1$ mice differing from the donors by only weak H factors, the survival of a subsequent transplant of skin was proportional to the time the ovarian graft was in place (Linder, 1962). This is suggestive of the establishment of unresponsiveness in the host, or of modification of antigenicity of the graft; both are highly unorthodox speculations.

2) *Antigen "Z".* Hoecker and his colleagues have described an antigen "Z" (Hoecker and Pizarro, 1962). The antibody against Z was highly reactive and Z seemed to be a typical H-2 antigen until a backcross was tested, when 15 per cent recombination between Z and S (a known H-2 antigen) was found. Little more is known about Z; it is apparently on the IXth linkage group but remote from H-2. It remains possible that other "H-2" antigens (*e.g.*, R) will later be found to be strung out along the chromosome (Hoecker and Pizarro, 1962). A widespread separation from H-2 appears unlikely from a limited study of the linkage of R to other H-2 antigens (Amos, 1962).

3) *Other Hemagglutinogens and Cytotoxinogens.* Several hemagglutinins reacting with factors which segregate independently of H-2 have been described (Amos, 1959). Contrary to general belief these antibodies may be quite strong and react with titers of over 1/2,000. Two antigens, at first described as $a$ and $\delta$, have been redesignated H-5.A and H-6.A and were mentioned above; the others are still being investigated under some difficulty owing to the undue instability of the antibodies to freeze drying.

Cytotoxic antibodies have been detected in several sera produced between animals of similar H-2 genotype. Two of these have been studied in some detail. C3H/He anti-C3H/St contains an antibody independent of anti-H-5.A, which reacts with tissues and tumors of several strains which do not carry H-5.A. Only one component has so far been detected. C3H/St anti-C3H/He, on the other hand, contains as many as 4 cytotoxic antibodies, two of which have been detected with some certainty; one of these reacts with antigen 12 on BP8 as mentioned above. Another is of considerable interest since the antibody produced in C3H/St mice reacts with a tumor, 6C3HED, indigenous to C3H/St mice. No reaction with C3H/St normal tissues has been found. The existence of this antigen provides a partial explanation for the earlier observations of Kidd that guinea pig serum had a marked inhibitory effect on the growth of this tumor in C3H mice (Kidd, 1953).

This series of antibodies is also of interest because the antibodies are produced between two sublines of the same strain. A study of their properties offers the opportunity of assessing the relative importance of as many as 7 antigens (2 hemagglutinogens and 5 cytotoxinogens) on a generally similar genetic background.

4) *Hybrid and "Recessive" Antigens.* The concept of hybrid antigens is of some antiquity, but with the possible exception of that described by Tyzzer (1916), hybrid antigens have not yet been detected in mice despite sporadic and generally unpublished searches. Deletions appear to be fairly frequent in tumors (Hauschka and Levan, 1953; Levan, 1959) and evidence has been accumulating that not infrequently a hybrid appears to be capable of reacting against one but not the opposite parent, possibly as a result of a deletion or of the repression of an antigen.

Results have been published by Popp (1961), by Cudkowicz (1961), by Celada and Welshone (1962), and by Glynn and his colleagues (1963) in support of the observation of $F_1$ anti-parent reactions.

Final evaluation of these results will take some time but the question is of the greatest interest especially in cancer research. The interpretation of results is made difficult by the weakness of the reaction, the use of non-pedigreed mice in most of the experiments, and the fact that any heterozygosity in the stock would be magnified in the $F_1$.

## Histocompatibility Factors in the Rat

Although sporadic reports of cytotoxic and hemagglutinating effects of isoantibodies in the rat have been published since before 1930, most of the current work dates only from 1960.

Lumsden (1937) and Phelps (1937) both described the cytotoxic effects of isoimmune rat serum and Lumsden (1938) published some hemagglutination studies, but this work was not pursued. Burhoe (1947) recorded several antigens of which one, "A," was well documented.

Owen (1962) recorded five factors detected as hemagglutinins following heterospecific immunization. One had an inverse frequency from Burhoe's A and could have been an allele. Two extremely reactive factors were designated "C" and "D." Strong saline antibodies were readily made to trypsinized cells and these antigens have later been used extensively as markers for experiments to follow the survival of transplanted bone marrow cells (Odell, et al., 1957). Two other factors are reminiscent of some of the mouse iso-antigens, the relevant antibodies being both weak and unstable.

Frenzl et al. (1960) and Bogden and Aptekman (1962) have independently used the designation "B" for an antigen. Palm has suggested that the former be known as B-1 and the latter as B-2 (Palm, 1963).

Whereas C and D had no effect in transplantation immunity and isoantibodies are not readily formed (Palm, 1962), both B-1 and B-2 are effective in iso-immunization and immunity to both resembles that against mouse transplantation antigens.

Immunity to B-1 has been tested extensively *in vivo*, and both runt disease and erythroblastosis fetalis have been attributed to immunity to this factor (Frenzl, Kren and Stark, 1960).

Anti-B-2 is a weak hemagglutinin in saline but reacts strongly in serum or dextran (Bogden and Aptekman, 1960). In the presence of complement, an antibody in the serum, possibly anti B-2, is strongly cytotoxic for ascites tumor cells. Animals carrying the controlling gene R-1 will accept a tumor possessing the B antigen. From backcross studies, the hemagglutinogen is a unit character, but Bogden and Aptekman suggest that there might be 3 allelic genes, $R\text{-}1^a$, $R\text{-}1^b$, and $R\text{-}1^c$. The evidence for this is inadequate and there is no confirmation that the hemagglutinogen, cytotoxinogen and transplantation antigen are determined by the same gene. The hemagglutinogen and the capacity to accept a tumor segregate as one factor, but there is still need for data relating to the cytotoxinogen. The hemagglutinogen was detected in 6 out of 15 inbred strains tested, and 3 noninbred strains were found to be heterozygous, with the majority of animals in each of three strains carrying the antigen. Four tumors

from the known "B positive" P.A. strain carried the antigen and one from the "B negative" Lewis strain did not (Bogden and Aptekman, 1960 and 1962).

It is clear from the studies of Billingham, Hodge and Silvers that the rat has as many histocompatibility antigens as the mouse (Billingham, Hodge and Silvers, 1962). Bogden and Aptekman (1962) noted the existence of several other antigens, one detected by tumor rejection following the immunization of P.A. rats with Wistar fetal tissue, and another detected as a cytotoxinogen on a P.A. tumor. The cytotoxin was produced in P.A. mice, a situation analogous to the detection of antibodies to 6C3HED produced in C3H/St mice.

A third factor, "G," was present in both P.A. and Lewis rats, while the noninbred Wistar strain was heterozygous for the antigen. Although the antigen is present on liver, spleen, tumor and red cells, it does not appear to be involved in transplantation immunity (Bogden and Aptekman, 1962).

Palm (1962) has described three additional factors and has given them the tentative designation 1, 2 and 3 until a definitive nomenclature can be adopted. The strain distribution of the three antigens together with data on antigens C, D, B-1 and G is given in Table 2.

TABLE 2—*Antigenic Characters of Four Strains of Rats*

| Strain | Presence of Antigen | | | | | | |
|---|---|---|---|---|---|---|---|
| | C | D | B | G | 1 | 2 | 3 |
| Lewis | + | − | − | + | + | + | − |
| B.N. | + | + | − | + | − | − | + |
| W.I.F. | − | + | + | − | − | + | − |
| P.A. | + | − | + | + | − | + | − |

Antigens 1 and 3 are believed to stimulate transplantation immunity since there is a close association between their presence and the acceptance of skin grafts in backcross hybrids. Although other antigenic factors are undoubtedly segregating in the crosses used, over 50 per cent of the rats carrying the pertinent antigen (1 or 3, respectively) retained their graft after 12 days whereas homozygous negative animals uniformly rejected the graft before 12 days. Hemagglutinins were present in the serum of the homozygous negative animals. Antigen 3 appears to be on the same linkage group as B-1 and could be an allele of it.

Thus, although for many years studies on the rat received considerably less emphasis than those in the mouse, the knowledge of the distribution of a series of antigens, C, D, B-1, G, 1, 2 and 3, among several representatives of the wide variety of inbred strains of rat now available should greatly accelerate the use of rats for transplantation studies. H-2 has stolen too much attention in the mouse and if other systems of comparable strength exist, they have not yet been recorded. The rat does not at present have a system of comparable complexity, but seems to have a variety of antigens, some potent and some of uncertain reactivity, useful in studies similar to those carried out on the murine model (Billingham and colleagues, 1962). Because of the lack of a single strong system like the H-2 of the mouse, studies on the rat may be more directly comparable to those on man where the emphasis is also on the existence of many factors.

## Man

While there are still considerable gaps, especially concerning the function of antigens and their relationship to differentiation and intracellular processes, the available information on the mouse H-2 is fairly impressive. Other systems have been considerably less intensively studied but information is beginning to accumulate. Yet, with regard to many antigens and antigenic systems in mouse, in rat, and above all in man, there is absolutely no information, not even the mere ability to recognize and identify them as transplantation antigens.

Although we cannot define one single human transplantation antigen with any certainty, several antigens appear to be very potent. Using the methods available for the detection of human iso-antigens, and with the animal studies as models, rapid progress is likely. While many renal and endocrine transplants have been performed, the amount of genetic information that has become available from these studies is small. Renal grafts are regularly accepted between identical twins (Murray, Merrill, and Harrison, 1958) and occasionally (and usually temporarily) between unrelated and genetically dissimilar individuals if the immune response is depressed. Knowledge of genetic interest available from these studies is still limited and discussion will be mainly restricted to studies of skin, white cells, and the fetus during pregnancy.

Skin transplantation has been actively studied for many years. Much of the earlier literature is of limited value because the grafts were of random size and thickness and the criteria for assessing graft rejection were not standardized.

Before 1910, skin homografting was frequently practiced, and grafts

were frequently thought to take. This belief was shown to be erroneous; a "take" was usually the result of replacement by cells from the recipient. Allogeneic isografts are now used only to provide temporary dressing in cases of extensive skin loss. Rogers (1950 and 1957) and Woodruff (1960) have given a summary of some of the earlier literature. Rogers has compiled a comprehensive bibliography of skin transplantation (Rogers, 1958). Rogers established that the rejection time of full thickness grafts in unrelated individuals was about 7-9 days, confirmed other reports that full thickness grafts would survive indefinitely in monozygotic twins and found prolonged survival (up to 29 days) in dizygotic twins (Rogers, 1957). Although there is some disagreement, Rogers believes that on balance ABO blood group compatibility was a factor, albeit a small one, in producing graft survival (Rogers, 1957).

For genetic studies, it is important to use a standard grafting procedure using normal recipients. Patients with severe burns, uremia, various malignancies, or with extensive skin loss reject grafts only after considerable delay. Differences due to antigenic factors may be superimposed on this background of delayed rejection. Longmire and his colleagues (1947) found that of 71 full thickness grafts applied to a single burnt recipient, most were not rejected until about the thirtieth day. One graft remained epithelialized after 5½ weeks; while this graft was still in place a split thickness graft from the same donor was put on a previously ungrafted area. The original and the second graft were rejected at the same time, some 9 days later. In this case one does not know if the rejection was due to an increased immunologic reactivity associated with improved health or was a second set phenomenon. An extreme example of prolonged graft survival in a burnt patient was given by Kay, who reported survival of a large graft up to 8 months, although other grafts applied at the same time were more rapidly rejected (Kay, 1957). Prompt and simultaneous rejection of grafts from the same donor placed on burned and unburned areas of a convalescent patient has been observed (Georgiade and Amos, unpublished).

Large grafts in man, especially when associated with removal of lymph nodes (Stark, Dwyer and DeForest, 1960) show delayed rejection. In the mouse a large graft may persist indefinitely, or be associated with patchy, chronic ulceration, if the genetic difference between donor and recipient is small (Martinez, Shapiro and Good, 1961). This has important immunological and therapeutic consequences, but the genetic analysis of skin antigens is so complex that it is better to avoid the question of large grafts and consider the evidence from small grafts made under conditions where sepsis and other complications do not further add to the difficulties. Too small a graft may be replaced rather than frankly rejected and

a graft of between 10 and 20 mm. in diameter seems to give the most consistent results.

Experimental studies have generally been confined to split thickness grafts to avoid scarring of the donor. Taylor and Lehrfeld, and Converse and Rapaport have described the use of the stereomicroscope to follow the vascular changes occurring in the graft, rejection being preceeded by dilation and thrombosis (Taylor and Lehrfeld, 1955; Converse and Rapaport, 1956).

Converse, Rapaport, Lawrence and Thomas set out to determine if certain antigens could be shown to be common to different individuals. The first step was to redefine under standard conditions the rate of rejection of a graft between unrelated individuals. It was established that the normal range is between 8 and 12 days with extremes of about 6 and 18 days. A second graft from the same donor placed some 10 days after the first graft has sloughed is rejected rapidly, the mean rejection time being 4.7 days (Rapaport, Thomas, Converse and Lawrence, 1960). If the second graft is placed within 5 days of rejection of the first, the host reaction is modified. The graft never becomes vascularized and forms an avascular sheet, the so-called white graft (Rapaport and Converse, 1958). White graft rejection is more difficult to assess than either a typical first or second set since the usual vascular changes cited by Converse and Rapaport (1956) and Taylor and Lehrfeld (1955) do not occur. The graft, at first white and avascular, becomes opaque and brown in about 6 days.

Nine subjects were given a sensitizing skin graft. Five days later they were given a second graft from the same donor and a graft from an unrelated subject. While the second graft from the original donor was rejected by a white graft reaction, the indifferent graft healed in. Three of the grafts survived normally for 8-9 days but five were rejected by the sixth day. This study gave evidence of antigenic overlap since five out of eight grafts underwent accelerated rejection. Rapaport et al. later extended these observations (Rapaport and colleagues, 1962). They argued that when sensitization of recipient A to skin from donor B would cause accelerated rejection of skin from unrelated donor C, subjects B and C must share antigens not present on the original recipient. Grafts were then performed between B and C, and as control, grafts were exchanged between donors where no accelerated response had been observed in the original test. When B sensitized A to C, grafts between B and C were accepted for 12 to 14 days. Where B did not sensitize against C, the survival was 7-9 days. This was a very small scale experiment, the values were within normal limits, but the results are suggestive and the method is a model for future experiments.

While these experiments provided evidence for some degree of antigenic overlap between skin from unrelated subjects, Lawrence and his colleagues have shown that it is possible to sensitize for skin graft rejection by means of prior injection of leukocyte extracts (Lawrence, 1960). Indeed these investigators have performed a number of experiments in which they were able to transfer skin graft sensitivity from one individual to another by means of cell free extracts (Lawrence, Rapaport, Converse and Tillett, 1960).

Merrill and his colleagues have demonstrated that subjects rejecting a skin graft show delayed hypersensitivity to leukocyte suspensions, that leukocytes will sensitize a recipient against a subsequent skin graft and that there must be many shared antigens between white cells and skin.

In a study by Friedman et al. (1961), two subjects were immunized with subcutaneous and 16 with multiple intradermal inoculations of white cells. Doses totalled $2.0 - 48.0 \times 10^7$ cells. Grafts from the leukocyte donor and from an indifferent donor were placed 6 days after leukocyte inoculation. There was no reaction in the two patients with subcutaneous inoculations, but 15 of the remaining 16 subjects gave an accelerated or a white graft rejection, the exception being in one of the two recipients receiving only $2 \times 10^7$ cells. Thirteen subjects showing immunity to the leukocyte injection received an indifferent graft. Of these, nine showed accelerated rejection and two gave a suggestion of enhancement since the grafts persisted for 21 days, well outside normal expectation. In contradistinction to the experiments of Rapaport et al., all recipients with a white graft reaction to the specific stimulus showed accelerated rejection to the indifferent donor and three out of eight gave a white graft reaction to him, indicating that this form of immunization is even stronger than that afforded by skin graft rejection.

The system proposed by Rapaport et al. (1962) will demonstrate that B and C both have marked antigenic divergencies from the recipient A. The model of Wilson et al. (1963) goes further and will pick up a donor with at least one strong antigen shared with the future recipient. Neither test is capable of excluding the possibility that the putative donor has one or more strong antigens lacking in the recipient, and in neither test is the indifferent recipient a neutral vehicle since his own antigenic constitution must influence his immune response. Two assumptions are inherent in both tests: (a) that the response to a graft is not affected by the response to other grafts placed simultaneously on the same recipient, and (b) that because the putative donor shares antigen(s) with the future recipient, the chance that he will have antigens which are not present on the recipient are appreciably reduced. Both can well be tested in animals, for example in mice, where the test could be used to try to

determine which individual from an $F_2$ population most nearly resembled one of the parent lines.

Hamburger et al. (1962) used close relatives as kidney donors and determined the reactivity of the leukocytes of donors and recipients against a variety of antileukocyte sera. Six donor-recipient pairs were tested. In three of these, either donor and recipient or recipient alone reacted with the sera used, and two of these three had the longest graft survival (with treatment). The series is small and the result may be due to chance, or the sera may not have included reagents reacting with certain alleles. More intriguing is the possibility that genes controlling leukocyte antigens like the ABO blood groups have allelic forms which are amorphic and do not produce effective iso-antigens.

The demonstration of antibodies in patients undergoing skin graft rejection is being investigated in a number of laboratories. Pavkova and Dolezalova (1962) have reported finding antibodies in 15 out of 16 skin grafted patients using agglutination of collodian particles coated with a simple saline extract of skin as indicator. This report is of interest but awaits confirmation. Sensitive *in vitro* methods of antibody determination have generally been avoided because the antigens are not yet defined and there is a considerable possibility of missing reactions by the arbitrary use of extracts of cells, or of being misled by false positives.

From skin transplantation studies it is clear that white cells share many common antigens with skin and platelets. Antibodies to white cells can be detected *in vitro* and it is therefore possible to proceed with a genetic analysis of white cell or platelet antigens in the expectation of being able to obtain a correlation with the antigens of skin and other tissues. The precedent for this is the correspondence between white cell and tissue antigens of the mouse and rat. Iso-antibodies to white cells have been known for many years (Wichels and Lampe, 1928). Dausset (1954) and Moeschlin and Schmid (1954) pioneered studies on auto-immunity to white cells, and Dausset (1954), Brittingham (1957), and many others, have shown that antileukocyte antibodies are often present after transfusion, especially following transfusion reactions not due to red cell incompatibility (Killmann, 1960; Walford, 1960). Payne (1957) found a good correlation between the incidence of antileukocyte antibodies and the number of transfusions. Antibodies develop in about 5 per cent of patients receiving 10 transfusions and over 10 per cent of those receiving 50 or more transfusions. Surprisingly, the majority of patients receiving over 100 transfusions do not have reactions and have no demonstrable antibodies. Unfortunately, because of the antigenic diversity of the donors, most of the post-transfusion antisera are a mixture of many different antibodies and attempts to absorb out some and leave specific

antibodies for use as reagents have usually been disappointing. Some sera are exceptional and have been used without absorption. Through the use of serum from multiply transfused recipients, Dausset (1957) showed that seven out of 27 sera gave the same reaction pattern on a test panel and were considered to be reacting against the same antigen, identified as MAC. Deliberate attempts to produce anti-MAC usually produced antibodies with a certain amount of cross reactivity (Killmann, 1958) and this method is now little used. The author is, however, currently investigating the properties of an antibody kindly provided by Dausset, which reacts with cells from only 8 per cent of the population.

In 1958, Payne and Rolfs (1958) detected antibodies to leukocytes in the serum of pregnant women. The nature of the antigenic stimulus is unknown. It is presumably from products of conception since women with less than two children rarely show antibodies. The antibody could result from the passage of white cells, soluble antigenic products or even other types of cells such as fetal trophoblasts or nucleated red cells, into the maternal circulation. Since there is generally only one father the number of antigens contributed by him is limited and the specificity of the antibody is likely to be greater than that obtained after transfusion with many dissimilar donors. Rood tested 2,500 sera from pregnant or postpartem women and analyzed 64 of these against leukocytes from a panel of 100 donors( Rood, van Leeuwen and Bosch, 1962; Rood, van Leeuwen and Eernisse, 1961). There was considerable specificity. Whereas Walford (1960) found that of nine multiple transfusion sera tested only one failed to react with most test suspensions (one serum reacted with 17 out of 18 cells tested), Rood found that most of his sera failed to react, or reacted only weakly, with many of the test cells. Twelve out of 64 sera tested intensively gave similar reactions. From this he concluded that the antibody was directed against one common antigen, which was designated leukocyte group "Four." By absorption of other sera it was possible to define two further groups but group four has been most constantly investigated. Two alleles have been identified, 4a and 4b, and from family studies it has been shown that these segregate as unit characters. The antigen is found in skin, platelets, kidney and placenta, but has not yet been implicated in transplantation reactions (Rood, 1962). Two other alleles, 5a and 5b have recently been identified and one allele 6a of a further system is being investigated (Rood, personal communication).

Payne and Hackel (1961) examined sera from a large number of multipara and selected eight antisera for intensive study. First they established the gene frequencies, assuming two alleles for each of the eight genetic systems; they then tested the sera on a series of families. In each

case the children of homozygous negative matings failed to react, and in the matings negative x positive or positive x positive the proportion of children reacting with the relevant antibody gave an excellent fit for the calculated gene frequency. Payne and Hackel appear to have demonstrated no less than eight antigenic factors and to have shown that each is inherited as a single dominant (? co-dominant) factor. Four of the factors show significant interrelationship but do not appear to be closely linked from consideration of individual families. In discussing this Payne and Hackel suggest either that there is multiple allelism for some of these factors or that the sera are polyvalent.

If the antisera used by Payne and Hackel were chosen at random, the gene frequency over a large series should approach a mean of 0.5. The mean frequency of the detectable allele in their series is 0.35. This suggests that they deliberately chose sera that were not widely reactive and this in conjunction with the family studies, suggests that their sera were predominantly specific. There is, however, no proof of this and their sera were not shown to be monovalent by absorption.

Certain criteria need to be laid down before a reaction can be ascribed to the reaction of antibody with a single specific antigen. The antibody must be shown to lose all reactivity after absorption with any cells carrying the antigen. It is also highly desirable that the allele be demonstrated, since confusion may result if the gene exists in more than two allelic forms. The definition of an antigen should also include a demonstration that individuals producing the antibody do not themselves react with it, nor should their cells absorb the antibody, and wherever possible studies on inheritance should include complementary tests with antisera against each allele.

Although antigenic analysis of white cell groups is proceeding there are difficulties in interpretation of the results. Most papers on white cell antigens do not mention the difficulties of the techniques which are still considerable. Several methods for detecting antigen are available; most frequently used are agglutination and antiglobulin consumption. Many modifications of the white cell test exist (see Killmann, 1960), the two major variants being in the original step, defibrination or the use of anticoagulant. The means used to get rid of the red cells, the medium for suspending the white cells, the cell concentration in the test, treatment of the tubes and heating of the serum are all sources contributing to the variation in sensitivity and in the detection of false positives. On theoretical grounds antiglobulin consumption should be more comprehensive but in practice fails to detect many strong reactions picked up by agglutination. Complement fixation is impracticable on a large scale because of the insensitivity and the large quantity of antigen required. Similarly the

localization of isotopically-labelled antibody is difficult to detect because of the high nonspecific adsorption of other globulins. These technical problems are serious and the routine use of methods for detecting antibodies to white cells is likely to be delayed until a simple and reliable method, preferably with stable suspensions of white cells, is available. The modification used in our laboratory appears to be sensitive and to give a low proportion of false positive results. Five ml. of blood is collected into 1 ml. of Plasdone C and 0.5 ml. of 5 per cent EDTA. The blood is mixed and allowed to stand until the red cells sediment. While the supernatant is still pink it is removed, centrifuged at 700 rpm for 10 minutes, the sediment resuspended in 0.1 ml. of the supernatant and allowed to resediment. The supernatant now contains a suspension of white cells with some platelets and very few red cells. The test is carried out at room temperature in 6 x 50 mm. tubes. 0.025 ml. volumes of serum dilutions in 1 per cent EDTA in phosphate buffer pH 7.0 are mixed with 0.025 ml. volumes of cells at a concentration of 1 x $10^7$/ml. The tubes are allowed to stand for 2 hours, the cells are drawn up and gently expelled into the tube, then transferred to a 3″ x 1″ slide. After standing for 30″ the slide is rotated for a few moments and the test read microscopically. All glassware is cleaned in chromic acid, carefully rinsed and siliconized. The use of siliconized tubes for incubating the test does not appear to diminish sensitivity appreciably, and the cells remain in excellent condition; false positives are rare except after very prolonged incubation (Amos and Peacock, 1963).

The principle limitation of this type of technique is that the cell suspension must be used within a few hours of collection, thus exchange of cells between laboratories is at present impracticable. Red cell grouping has been aided greatly by the ability of red cells to stand shipment and prolonged storage; white cell grouping will advance much more rapidly when white cells can also be preserved. Preservation of cells in liquid nitrogen after the addition of 10 per cent dimethyl sulphoxide has been reported by several authors, Rowe and his colleagues (1963), for instance, report that agglutinability is unaffected by this method of preservation. The method is simple, and if it is found to give reliable results in routine use, much of the difficulty in comparing results between different laboratories will disappear.

The problem of relating leukocyte antigens to transplantation immunity is not resolved. From the preliminary studies already published, it is obvious that there are multitudinous white cell groups. The greater frequency of white graft or accelerated reactions to an indifferent donor following preimmunization with white cells compared to the relative infrequence of strong cross-reactions after a skin graft appears to be related

to the difference in intensity of immunity and reflects the great range of antigens on the white cell. Another indication of this comes from experiments on erythroblastotic infants transfused at birth. Woodruff attempted to induce tolerance in human infants at birth but the results are inconclusive (Woodruff, 1957). Peer has published an account of the results of grafting between mother and infant (Peer, 1957, 1958). In several instances maternal skin survived for considerable periods. This would sugges that the child was tolerant since skin from the father was characteristically rejected rapidly. The child's skin also occasionally persisted on the mother, but it is not known if this was an artifact because of the nature or size of the graft; considerable genetic similarity between mother and child; or even possibly enhancement by antibody produced by the mother against the fetus similar to the enhancement of tumor growth by antibodies reported by Casey, Snell, Kaliss and others in mice (see Kaliss, 1962). A somewhat analogous experience to that of Peer has been reported in mice by Breyere and Barrett (1960). These authors found that inbred female mice mated with a male of a different strain would accept a tumor from the male strain. Since human mothers frequently produce antibodies against the fetus, the anomalous results of Breyere and Barrett as well as those reported by Peer and the observations quoted earlier of Linder (1962) on ovarian transplants could perhaps be best explained by postulating the induction of enhancement rather than of tolerance. Lejeune has reported that erythroblastotic infants given an exchange transfusion at birth may be tolerant not only to the specific donor of the fresh blood used for transfusion but also to an unrelated individual of similar red cell type (Lejeune, 1962).

Eight children aged between 3 and 7 years who had received exchange transfusions at birth were grafted with skin from one of the putative blood donors. In four patients there was prompt rejection, in two slow rejection, and in two prolonged acceptance. The slow rejection took from 3-6 months, and in one of these the skin was mistakenly taken, not from an actual donor, but from another individual of similar red cell type. One of the grafts for which acceptance was indefinitely prolonged was from an elderly man. After 5 years the graft was shown to the author who was impressed by the typical wrinkling and atrophic appearance of a patch of aged skin on the arm of a young girl. Unfortunately, permission for biopsy to test for the sex of the skin could not be obtained. In the other case of prolonged survival, two grafts were placed, one from a former blood donor, the other from a dizygotic twin of the patient; both survived. Similar additional studies are urgently needed, especially to confirm and investigate the apparent tolerance, the lack of specificity as shown by lack of reaction against the two skin donors who had not

given blood and the abortive reaction manifested in two cases on about the twenty-first day after which the graft again appeared to be accepted (Lejeune, 1962). A similar over-all proportion of subjects accepting grafts for extended periods was reported by Fowler et al. (1960), but these authors found that infants given fresh blood were tolerant to skin from the donor, whereas children who had received stored blood had only a feeble suggestion of tolerance to the mother.

While the studies of Lawrence, Merrill and their colleagues have shown that skin and white cells share many antigens and the observations of Payne and others have made it possible to obtain a rich variety of anti-leukocyte antibodies, many questions concerning the correlation between the antigens of skin and white cells remain unresolved. At face value the observations of Peer and of Lejeune indicate that in certain instances white cells carry all the pertinent antigens. These reports remain largely unsubstantiated and further experiments are urgently needed. If confirmed, then the problem of relating leukocyte to transplantation antigens becomes greatly simplified. If not, the only reconciliation between leuko-agglutinogens and transplantation antigens appears to be through painstaking attempts to match skin donor and recipient for a steadily increasing number of antigens.

A review of transplantation immunity when so much remains unknown is frustrating to the reader and to the writer. An attempt has been made to weave a skeleton pattern of the general subject. Isolated areas have been studied in great detail, especially with respect to the genetics of bone marrow transplantation after X-irradiation and these have not been mentioned. Nor has any attempt been made to cover the literature in its entirety, since the present need is for more competent investigators to enter the field rather than for another documentation of an incompletely studied field. My aim is to point out the need for many more studies on the genetics of transplantation antigens and to show that the area is ripe for exploitation.

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Chapter 5

# The 21 Trisomy—Current Stage of Chromosomal Research

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## History and Terminology

In 1844 and 1846, seguin described a particular type of mental retardation which he called, "furfuraceous idiocy." His description is still considered by such specialists as Benda (1962) to be the "most ingenious description of the physical characteristics of the mongoloid growth deficiency."

The term "mongoloid" was introduced later, following the racial hypothesis advanced by Langdon Down (1866) who suggested that the mental deficiency was related to the resurgence of traits of the mongolian races. Fortunately, other types of mental deficiencies which he classified as negroid and Malaysian, have been discarded from medical nosography; mongolism, however, still persists.

A recent proposal for avoiding the term mongolism was made by Allen et al. (1961), who suggested the names "Langdon Down syndrome" or "21-Trisomy." Unfortunately, "Langdon Down Syndrome" would represent the consecration of both a historical error, since Seguin was the first to describe the condition, and an etiological error, since the additional chromosome which causes the disease has no relation to mongolian races. In the present review the disease will be referred to as "21-trisomy" and the patients as "21-trisomics." Regardless of the standards of its recognition, the 21 chromosome is by definition the chromosome which produces "mongolism" when present in triplicate. The use of such a specialized

word may be premature, but the discovery of etiological agents has always led to the renaming of diseases. Such accepted terms as "staphylococcemia" and "hypoparathyroidism" seem no longer difficult to understand, although they were undoubtedly considered unprepossessing at the time of their introduction.

## Clinical Features

"Furfuraceous cretinism, with its milk white rosy and peeling skin, with its short-comings of all integuments, which give an unfinished aspect to the truncated fingers and nose; with its cracked lips and tongue, with its red, ectopic conjuctive, coming out to supply the curtailed skin at the margins of the lids" (Seguin, 1866) . . . is a quite obvious syndrome. The mask of imperfect definition thrown on the bodies of the children, makes them so alike that "il suffit d'avoir vu l'un d'entre eux pour ne plus risquer de les méconnaitre" (Turpin, 1931).

Aside from this particular aspect, the major symptom of the disease is mental deficiency. Although carefully studied by many investigators, the 21-trisomics do not basically differ from other feeble-minded persons, at least from the point of view of inteliigence tests. Dunsdon et al. (1960), have recently stated that the I.Q. of these children is rarely greater than 80 per cent. During the first years of life, however, the I.Q. may frequently be as high as 70 per cent.

The progressive regression of the I.Q. of 21-trisomics is exemplified by the large study of Øster (1953) and has been elsewhere discussed (Lejeune, 1960). This deterioration has recently been confirmed by Zeaman and House (1962). The morphological picture of the 21-trisomic children and the associated malformations are too well known to be reiterated. For a clinical review, the reader is referred to the monographs of Øster (1953) and Benda (1960). We will attempt to review the multiple components of the syndrome in so far as they throw light on its occurrence, its nature, and its consequences.

### *I. Epidemiology of the Disease*

*Frequency.* The 21-trisomy is a relatively common disease, possibly the most frequent of all congenital anomalies.

Table 1 summarizes current data on its frequency at birth, which is of the order of 1/600. There seems to be no difference in frequency between mongolian populations (Japan and Hawaii) and white populations (Europe, America, Australia).

TABLE 1

| Authors | Region | Frequency |
|---|---|---|
| Jenkins (1953) | Chicago | 1/636 |
| Malpas (1937) | Liverpool | 1/776 |
| Carter and MacCarthy (1951) | London | 1/666 |
| Øster (1953) | Zeeland | 1/765 |
| Collman and Stoller (1961) | Australia | 1/688 |
| Schull and Neel (1962) | Hiroshima and Nagasaki | 1/785 |
| Wagner (1962) | Honolulu | 1/478 |
| Jaworska (1962) | Poland | 1/574 |

*Maternal Age Effect.* Maternal age was the first factor recognized to be important in the etiology of the disease. Shuttleworth observed in 1895 that nearly half of the patients were the last-born of a large family. In 1909, he suggested that the aging of the mother had probably an unfavorable influence, and considered the 21-trisomics as "exhaustion products." Since these early observations, numerous authors have studied the disease; the effect of maternal age has been the only point of agreement in etiological research on 21-trisomy before the recognition of the chromosomal aberration.

Paternal age, as well as birth rank, have been demonstrated to have no influence (Jenkins, 1933; Penrose, 1933 and 1934). Recently, paternal age has been implicated in the rare cases of transmission of 21-22 translocations, (Penrose, 1962); however, it is only the maternal age effect which seems to be of statistical importance. Relevant data on this point have been presented by Penrose (1961) and by Carter and Evans (1961). The graph shown in Figure 1 is based on data of Carter and Evans. It should also be pointed out that a maternal age effect has been observed in two other trisomies (17-trisomy and 13-trisomy) and is of the same order of magnitude (Lejeune, 1962).

*Clinical Aspects.* The characteristic clinical features of the disease suggest that 21-trisomic children are affected from head to foot, in that every part of their body shows small departures from normal. It is the accumulation of these deviations, each of which is often at the borderline of the normal, which permits the diagnosis of the disease.

Certain peculiarities are especially significant.

*Dermatoglyphic Traits.* There is a vast literature on the dermal ridges of the hands and feet of 21-trisomics. Extensive studies by various authors stress four traits which are thought to be typical of the disease (Crookshank, 1931; Penrose, 1954; Turpin and Lejeune, 1953b):

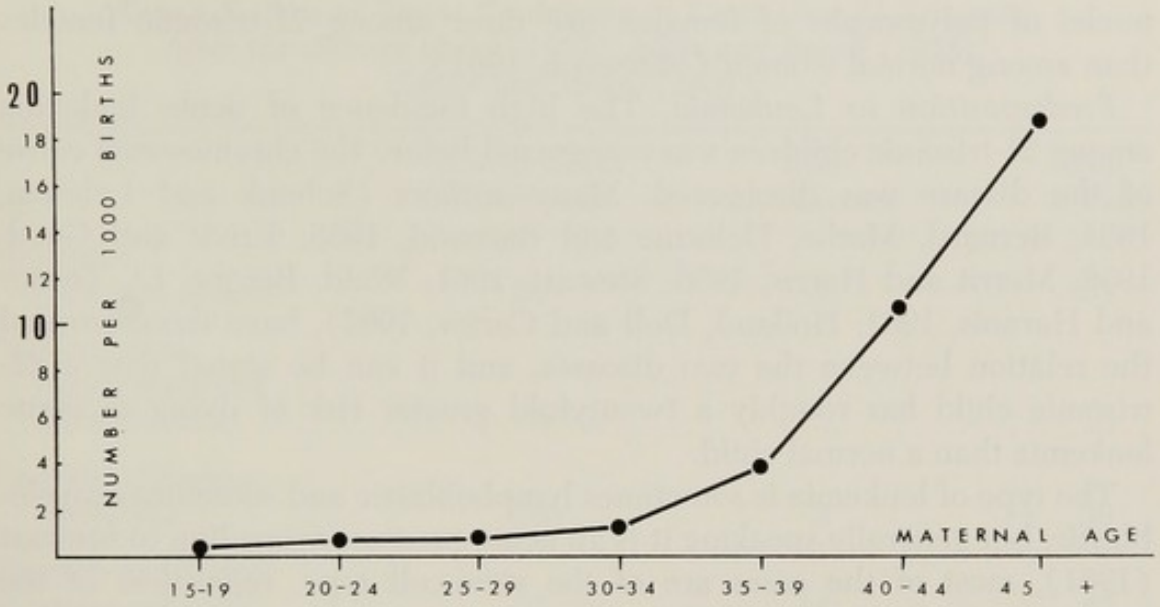


FIG. 1—Number of 21-trisomic children per thousand birth, relation with the age of the mother (drawn after the data of Carter and Evans, 1961).

1) The transversality of the ridges of the distal portion of the hand.
2) The medio-palmar position of the axial triradius (the t position).
3) The lunar loop on the hypothenar region.
4) The "simian line" crossing the palm transversely.

It has been shown (Turpin and Lejeune, 1954a and b) that these four traits have taxonomic significance, since they are typical of monkeys and therefore permit a discrimination between monkeys and the anthropoids.

The apparent resurgence of phylogenetic characters in 21-trisomy leads to the conclusion that a severe genetic imbalance can overcome genic modifications accumulated during species differentiation. Other trisomies, especially 13-trisomy, also show changes in the dermatographs (Uchida, Patau and Smith, 1962), and it appears that the genetic mechanism of these traits is based on a complex interaction between numerous genes. A review of dermatoglyphic changes in various imbalanced karyotypes leads to the same conclusion (Penrose, 1963).

*Nuclear Segmentation of Polymorphs.* Decreased nuclear segmentation of the polymorphonuclear leukocytes was the first cellular anomaly recognized in this disease. Described in 1947 by Turpin and Bernyer and widely confirmed since (Shapiro, 1949; Lüers and Lüers, 1954; Mittwoch, 1957 and 1961; Canevini and Maderna, 1962), this shift to the left of the Arneth count is definitely not related to the infections frequently observed in these children, but to a constitutional anomaly of the nuclei. In addition, the so-called "drumsticks," *i.e.*, the small appendices seen on the

nuclei of polymorphs of females, are rarer among 21-trisomic females than among normal women (Mittwoch, 1961).

*Predisposition to Leukemia.* The high incidence of acute leukemia among 21-trisomic children was recognized before the chromosomal cause of the disease was discovered. Many authors (Schunk and Lehman, 1954; Bernard, Mathe, Delorme and Barnoud, 1955; Krivit and Good, 1956; Merrit and Harris, 1956; Stewart, 1961; Wald, Borges, Li, Turner and Harnois, 1961; Holland, Doll and Carter, 1962), have demonstrated the relation between the two diseases, and it can be stated that a 21-trisomic child has roughly a twentyfold greater risk of dying of acute leukemia than a normal child.

The type of leukemia is sometimes lymphoblastic and sometimes myeloblastic, but generally speaking it is an acute process. According to Stewart (1961), most of the cases are of the stem-cell type, regardless of the actual morphology of the pathologic elements. Also, it should be recalled that the risk of congenital leukemia is extraordinarily high among 21-trisomics. This type of disease is extremely rare; although no final statistics can be given, the excess of 21-trisomics is very great among congenitally affected patients. It seems worth emphasizing that congenital leukemia has also been observed in a case of 13-trisomy (Schade, Schoeller and Schultze, 1962). This association between the 21-trisomy syndrome and the leukemic process and the abnormal segmentation of the polymorph nucleus suggests that these congenital malformations are fundamentally related to the underlying genetic cause of the disease.

*Genetic Aspects.* A genetic basis for the 21-trisomy disease was suspected long ago on the basis of three sets of data: twin data, reproduction of 21-trisomic mothers, and accumulation of the disease in exceptional families.

*Data on Twins.* Most of the available information in the literature is summarized in Table 2, which is based on the combined data of Øster (1953), Allen and Baroff (1955), and Carter and Evans (1961). The most striking finding is that dizygotic twins are as a rule discordant, and monozygotic twins always concordant. This result excludes a specific effect of the uterine environment. Nevertheless an exceptional case of monozygotic heterokaryotic twins has recently been observed (Lejeune, Lafourcade, Scharer, de Wolff, Salmon, Haines and Turpin, 1962), in which one of the twins was 21-trisomic and the other was normal. This exceptional type of twinning will be discussed subsequently.

*Reproduction of 21-Trisomic Mothers.* Very rarely 21-trisomic females procreate. Nothing is known about pregnancies of normal women fathered by a 21-trisomic male although there is no reason to suppose that 21-trisomic males are sterile. If unreliable observations are excluded, eleven 21-trisomic mothers have been reported in the literature (Table 3). As

TABLE 2—*Sets of Twins Containing at Least One 21-Trisomic. After the data of Øster (1953), Allen and Baroff (1955), Carter and Evans (1961)*

| | One of Them 21-trisomic | Both 21-trisomics | No. of Pairs |
|---|---|---|---|
| Dizygous (different sexes) | 59 | 0 | 59 |
| Monozygous (same sexes and identity tests) | 0 | 13 | 13 |
| Probably dizygous (same sex) | 36 | 3 | 39 |
| Zygosity not stated (same sex) | 33 | 14 | 47 |
| | 128 | 30 | 158 |

shown in Table 3, the affected mothers are relatively young (at birth of their children). Contrarily their own mothers were rather old at the birth of these trisomic mothers. This is in accordance with the maternal age effect previously quoted. Fifteen pregnancies from these mothers have been recorded. If monozygotic twins are counted as one zygote, these pregnancies may be divided into five 21-trisomics (including a still-born), and eight nontrisomics (including six normals, one mental defective and one still-born). Thus, there is fairly good agreement between these data and a 1:1 segregation of a dominant character. The ratio is in accord with the possibility of meiotic reduction in trisomics, who should produce in equal number normal (haplo-21) and abnormal gametes (diplo-21).

*Familial Occurrence.* Recurrence of the disease in a given family is a rare event. Most of the cases are isolated occurrences in an otherwise normal family, but the number of sibships including at least two 21-trisomics is significantly higher than would be expected by chance alone (Penrose, 1934; Øster, 1953; Carter and Evans, 1961). One exceptional instance includes a family of seven children, five of whom are affected (Turpin and Lejeune, 1953a). Such cases suggest existence of a particular mechanism for the disease. As will be discussed subsequently, the occurrence of a balanced translocation in one of the parents in these sibships explains the recurrence of the disease. Nevertheless, as pointed out by Hamerton, Briggs, Giannelli and Carter (1961), even if translocation cases are ex-

TABLE 3—*Progeny of 21-Trisomic Mothers*

| Reference | Age of the Mother | Age of the Mother's Mother | Father of the Child | Children |
|---|---|---|---|---|
| Sawyer (1949); Sawyer and Shafter (1957) | 25 | ? | Father of the mother (incest) | Normal girl; now a student 18 yr. old |
| Lelong et al. (1949) | 30 | 42 | Feeble minded (not 21-trisomic) | Boy, clinical syndrome of 21-tris.; dead 1 month |
| Rehn and Thomas (1957); Stiles and Goodman (1961) | 19 *(tris.-21)* | 30 | unknown | Girl *(tris.-21)* |
| Forssman and Thyssel (1957); Lehmann and Forssman (1960) | 20 *(tris.-21)* | 42 | Blind, epileptic *(46 chromosomes N)* | Normal boy *(46 chromosomes N)* |
| Schlaug (1957) | 29 | 39 | Father of the mother (incest) | Abnormal girl; syndrome very different from the classical 21-tris. |
| Hanhart (1960); Hanhart et al. (1961) | 21 *(tris.-21)* | | Brother of the mother (incest) | Girl—*(tris.-21)* |
| | 23 | 44 | Feeble-minded *(46 chromosomes N)* | Boy—Typical syndrome of 21-tris.; dead 1½ yr. |
| Mullins et al. (1960) | 22 | 22 | Feeble minded | Normal boy |
| Levan and Hsu (1960) | ? | ? | ? | Normal boy *(46 chromosomes N)* |
| Thuline and Priest (1961) | 14 *(tris.-21)* | ? | ? | Normal monozygous twin boys *(46 chromosomes N)* dead |
| Thompson (1961) | 21 | ? | Feeble minded | Normal boy, now 10 yr. old |
| | 20 | ? | ? | Female macerated foetus |
| | 22 | ? | ? | Male dead foetus probably 21-trisomic |

Note: The subjects for which chromosomal analyses are available are indicated: *(tris.-21)* or *(46 chromosomes N)*.

cluded, a small excess of recurrence still occurs in some families; these authors raised the problems of gonadal mosaics and the familial tendency to abnormal segregation. The accumulation of minor stigmata of the disease in healthy members of an affected family has also been reported, such as furrowed tongue (Turpin and Caratzali, 1933; Turpin, Bernyer and Teissier, 1947) and elevation of axial triradius (Penrose, 1954). The existence of hypothetical genes which increase the chance of mis-segregation of chromosomes should also be considered.

## *II. The Chromosomal Anomaly*

Speculations on the etiology of mongolism from 1846 to 1959 are so numerous that it is difficult to imagine a factor which has not been incriminated (see Warkany, 1960, for review). From the general data pointing toward a constitutional disease which acts on numerous traits two authors (Waardenburg, 1932; Bleyer, 1934) drew the formal conclusion that the disease was determined by a chromosomal aberration and accepted this etiology as the only one possible. These two remarkable papers show that in biology logical deductions may precede physical observation by a quarter of a century. Other authors have also discussed the chromosomal hypothesis to explain exceptional families (Penrose, 1939), or to explain the general data (Turpin and Caratzali, 1937; Fanconi, 1939). Nevertheless, the etiology of the disease remained unproved until the observation of the 21-trisomy.

*The Typical 21-Trisomy.* Prior to 1958 the only published attempt to observe the chromosomes of affected patients was the investigation of Mittwoch (1952); with the limitations of the available techniques, she was unable to arrive at a firm conclusion. She observed 24 chromosomal masses at meiosis and supposed that this was explained by the somatic number of 48, which was the accepted diploid number at that time.

Two years after the establishment of 46 as the correct somatic chromosome number of man (Tjio and Levan, 1956; Ford and Hamerton, 1956), an affected child with 47 chromosomes was observed in Paris. A further study of two other cases was completed in 1958 and the existence of an extra chromosome in this syndrome was published in January 1959 (Lejeune, Gautier and Turpin, 1959b). Additional confirmations were published by Ford, Jones, Miller, Mittwoch, Penrose, Ridler and Shapiro (1959), Jacobs, Baikie, Court Brown and Strong (1959), and Böök, Fraccaro and Lindsten (1959). Subsequently the 21-trisomy was demonstrated as the cause of the disease in all human races (Makino, Tonomura and Matsunaga, 1960; Lee, Schmid and Smith, 1961; Kleisner, 1961; Conen, Bell and Rance, 1962).

As exemplified in Figures 2 and 3, the presence in triplicate of a small satellited acrocentric chromosome can be established beyond any doubt in suitable cells of affected individuals (for technical discussion, see Lejeune, 1960). Many authors have discussed whether the triplicated chromosome is number *21* or *22*. In the "Denver agreement" it was stated that chromosomes should be classed in decreasing order of length; if satellites are excluded, chromosome 22 is slightly longer than 21, thus emphasises the difficulty raised by the numerical classification.

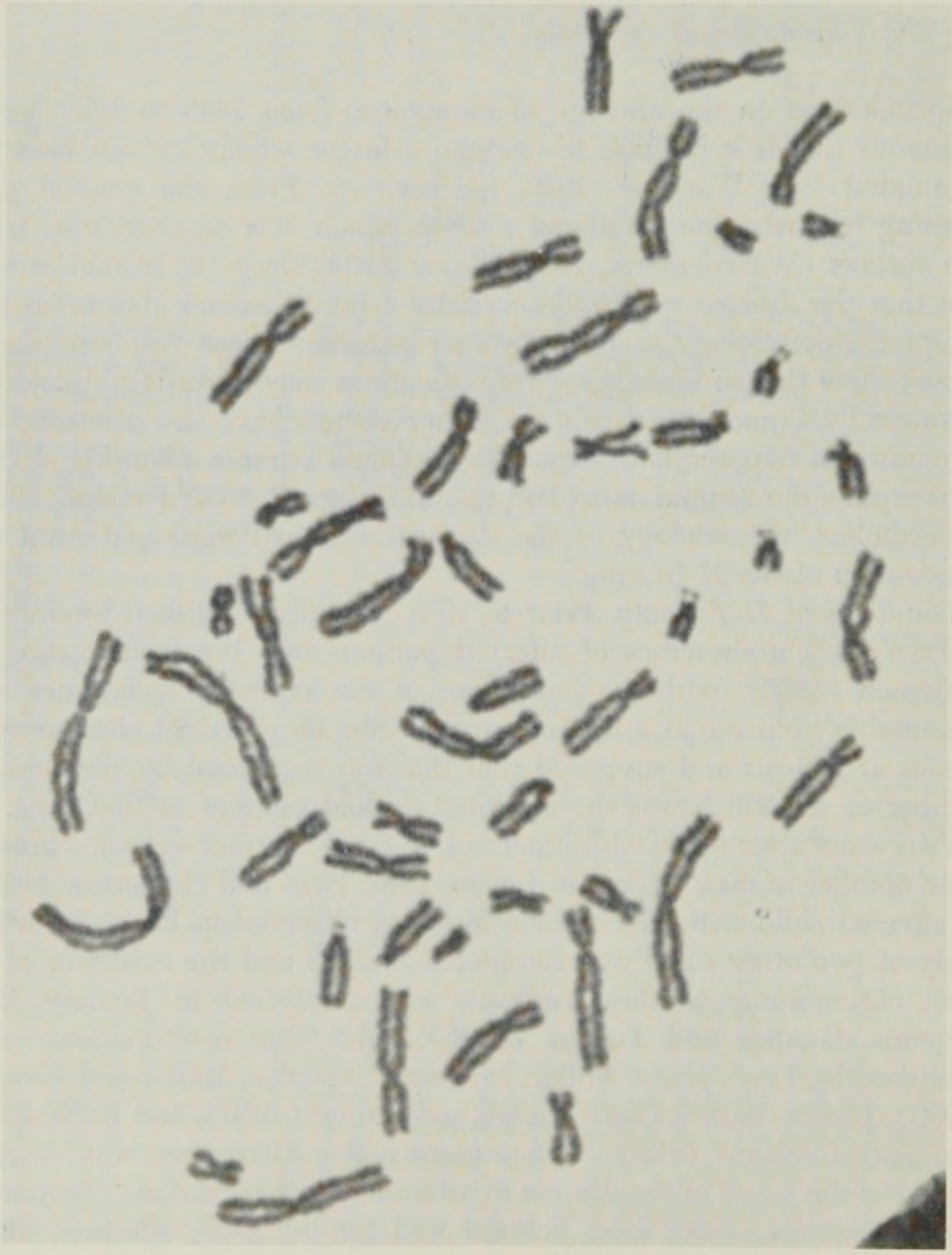

FIG. 2—The 47 chromosomes of a 21-trisomic male.

A definite position must be taken in this byzantinian quarrel; it can be presented as follows: The chromosome which produces the so-called "mongolism," when in triplicate, is to be called *21*. The other small acrocentric pair is then numbered *22*. It should also be pointed out, as accepted in Denver, that if new morphological characters were found which would aid in recognizing chromosome 21, they would not warrant a change of the already accepted number.

*The Trisomic State*. The observation of three similar elements is not in

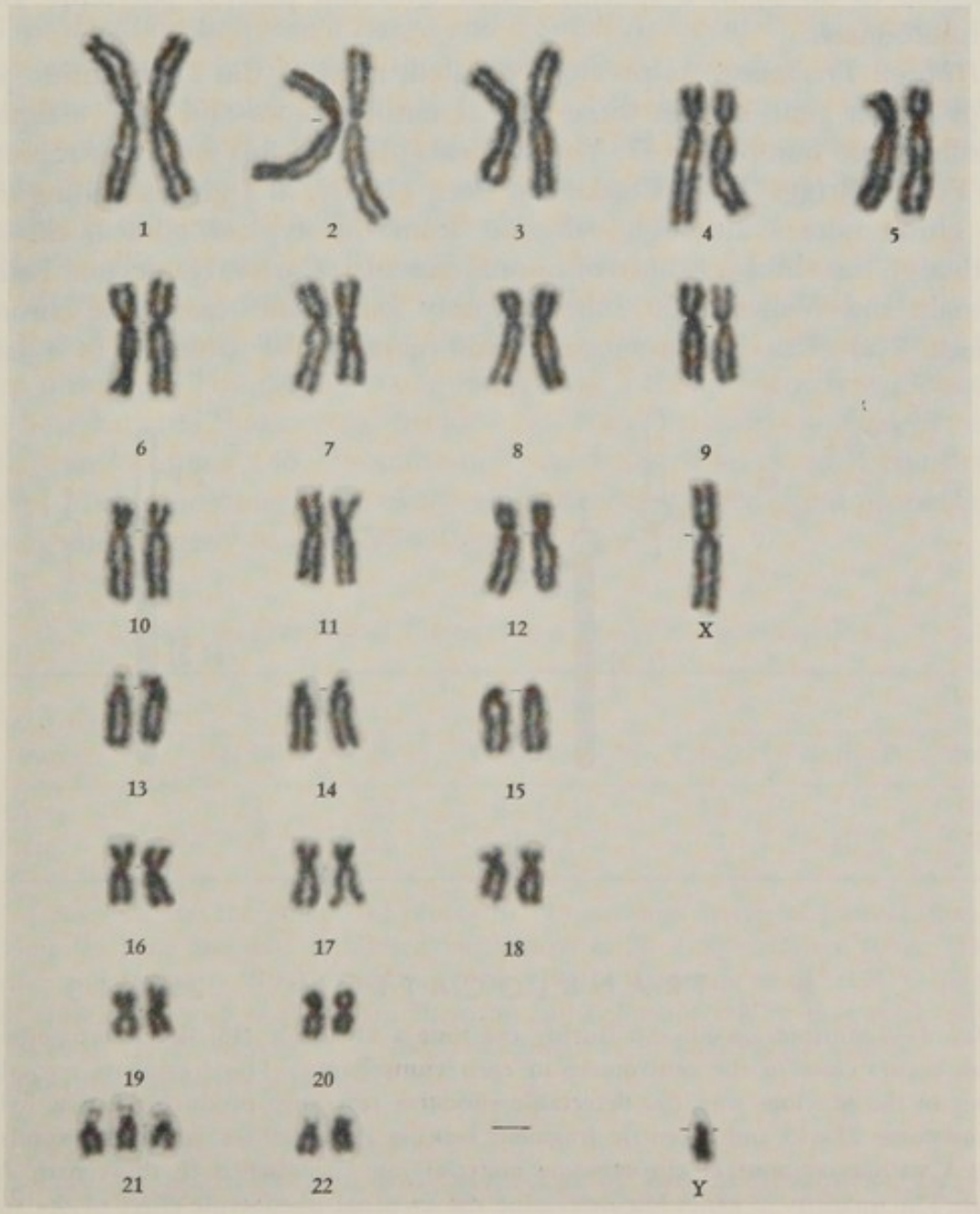


FIG. 3—Karyotype of the preceding cell. Remark: three small acrocentrics with satellites (21-trisomy). One of the 22 also exhibit satellites but less prominent.

itself sufficient reason for accepting the hypothesis that the elements are homologous chromosomes present in true trisomy. Fortunately, meiotic studies have been performed on testicular biopsies of 21-trisomic males. The production of trivalents has been recorded in a pure trisomic (Miller, Mittwoch and Penrose, 1960) and also in translocation carriers (Hamerton, Cowie, Giannelli, Briggs and Polani, 1961).

Since the synaptic process is the best indication of homology, it may be concluded that the trisomic nature of the disease is not only logically established, but that it has been confirmed cytologically as far as it is possible to do so in a species which does not possess polytenic giant chromosomes.

*Hidden Trisomies.* As previously stated, most of the 21-trisomics, perhaps 95 per cent, exhibit three free 21 chromosomes and have a diploid chromosome number of 47. The first exception to this rule was reported by Polani, Briggs, Ford, Clarke and Berg (1960), in a girl exhibiting only 46 chromosomes although suffering from the typical clinical disease. Although the child was born of normal parents (Carter, Hamerton, Polani, Gunalp and Weller, 1960), she had only four small acrocentric chromosomes. The extra 21 chromosome was found to be attached to a large

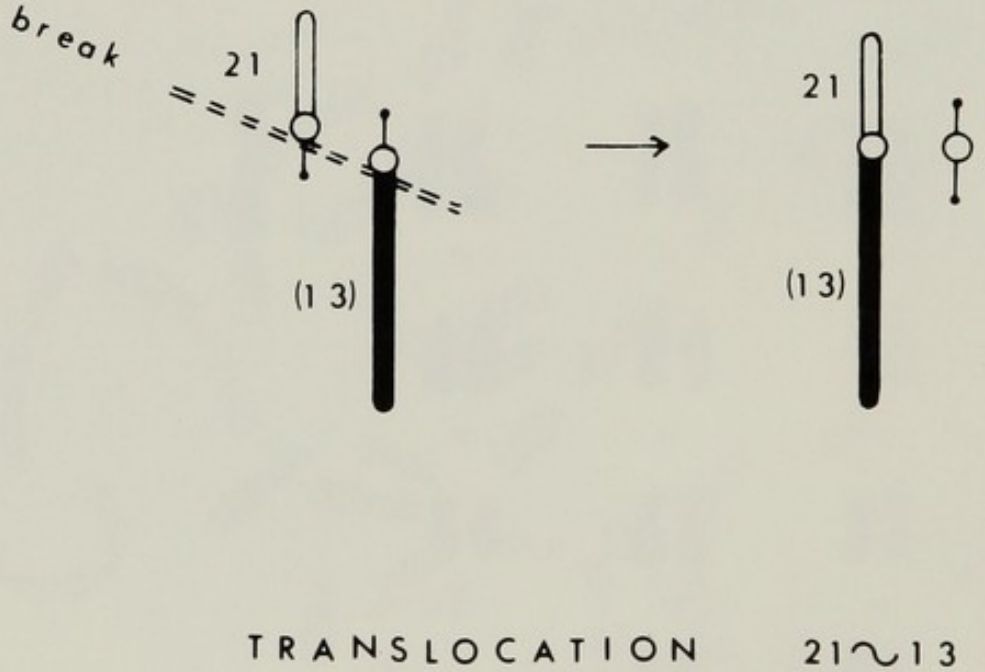


FIG. 4—*Translocation 21∿13.* During the time a 21 and a (13) lie close together, a break occurs close to the centromere of each chromosome. The fragments recombine either in the previous way (no detectable effect) or rearrange producing a new hybrid chromosome 21∿13 and a centric fragment bearing the satellites, which is secondarily lost. A various amount of chromosome material can be attached to the centric fragment. The quantity of genes lost determine the eventual phenotypic effect of the translocation. This general process of rearrangement between acrocentrics is commonly referred to by the rather misleading term centric fusion.

acrocentric chromosome of the 13-15 group. Such transfer of chromosomal material was very similar to the first case of translocation reported in man (Turpin, Lejeune, Lafourcade and Gautier, 1959), which also occurred between a small and a large acrocentric chromosome.

The number of instances of translocation in the literature is now significant. Their general importance has been previously discussed (Turpin and Lejeune, 1961), and their role in 21-trisomy has been recently reviewed (Carr, 1962). For the sake of simplicity they can be classified into two categories, those between a large and a small acrocentric chromosome and those between two small acrocentric chromosomes.

*Translocation Between a Large and a Small Acrocentric.* In general, the large acrocentric involved in this rearrangement (see Fig. 4) cannot be cytologically recognized, although it certainly belongs to the 13-15 group. For simplicity it will be referred to here as a (13) chromosome; the number in bracket refers to the whole class and does not specify whether the element is in fact a 13, a 14 or a 15 chromosome.

The small acrocentric is by definition chromosome 21, since it produces the 21-trisomy syndrome if in triplicate. Several families have been investigated in which the segregation of the rearranged chromosome has been observed. The data are summarized in Table 4. At a first glance it can be estimated that normal persons (46 chromosomes), normal persons with translocations (45 chromosomes), and 21-trisomics with translocations (46 chromosomes), are observed in approximately equal proportions among the progeny of carrier mothers.

TABLE 4—*Progeny of Carriers of a 21~(13) Translocation*

| Carrier Parent | Children | | | | | |
|---|---|---|---|---|---|---|
| | N.46 | N.45(T) | Tris.-21, 46(T) | Tris.-21* | Normal* | Total |
| Mothers: 32 | 23 | 26 | 25 (14) | 13 | 11 | 98 |
| Fathers: 15 | 15 | 22 | 1 (1) | 1 | 10 | 49 |

( ) No. indicates the number of propositi; (T) indicates carrier of translocation.

Compiled from the data published by Penrose et al. (1960); Carter et al. (1960); Penrose and Delhanty (1961); Buckton et al. (1961); Hamerton et al. (1961); German et al. (1962); Sergovich et al. (1962); Forssman and Lehmann (1962); Breg et al. (1962); Atkins et al. (1962); Biesele et al. (1962); MacIntyre et al. (1962) Shaw (1962).

* Only the phenotype is known.

It may be assumed that the reciprocal of the 21-trisomics (*i.e.*, the haplo-21 zygotes) are produced in comparable number but do not survive. In such a case the conclusion would be that once in every two

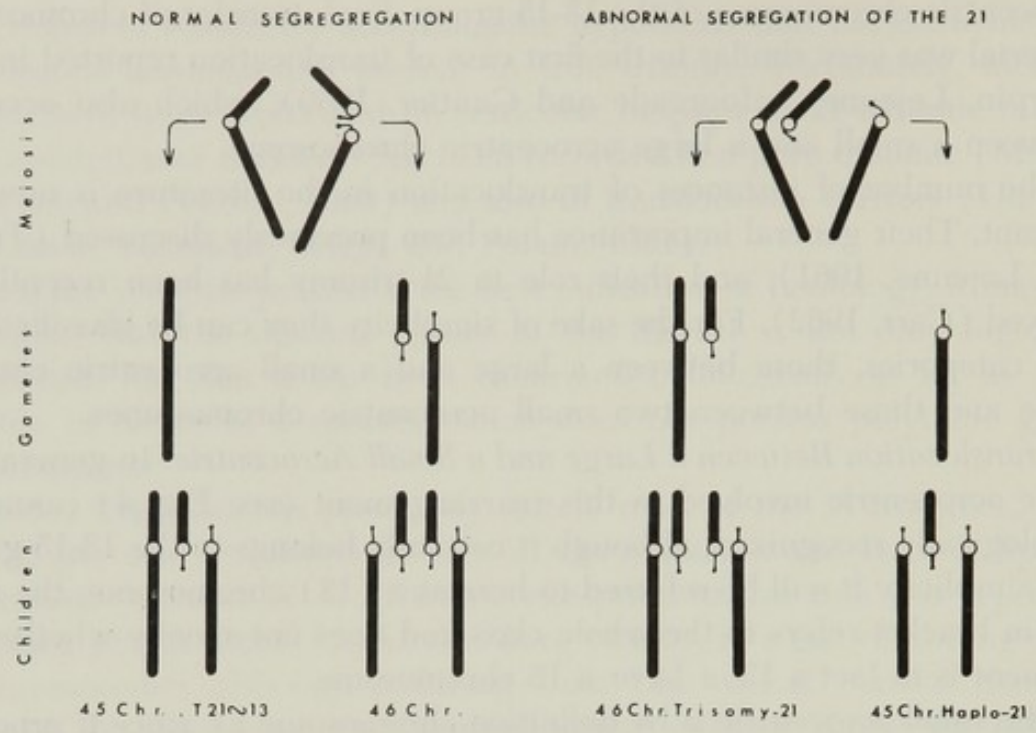


FIG. 5—*Progeny of a carrier of a 21~(13)* translocation. In the simplified diagram, of meiosis each chromosome is represented for sake of simplicity by one chromatid instead of two. The progeny show the four types of possible children of which only the three first are effectively observed, the type Haplo-21 being not known (probably lethal).

meioses, there is a mis-segregation of the free 21 chromosome. In other words, the translocation completely disturbs the assortment of the 21 chromosome, and the free one and the translocated one segregate independently (see Fig. 5). Table 4 is also useful for the purpose of genetic counselling, as it shows that a translocation carrier mother will as a rule give birth to a 21-trisomic child once in every three pregnancies.

The picture concerning the fathers is quite different, as is seen in the table; only two cases of trisomy-21, one of which is the propositus, are recorded among 49 children. The reason for such a difference between male and female carriers is not understood.

*Translocations Between Two Small Acrocentrics.* In this case it is difficult to tell whether the translocated chromosome is formed by the fusion of a 21 and a 22, between two 21's or even between two 22's. The recognition of the 21 is difficult in itself and becomes particularly troublesome in cases of translocation. The nucleolar material, divided among the satellites is the object of competition between the acrocentric chromosomes, as shown by Nawaschin (1934). Hence the use of the satellites as markers of 21 becomes misleading under these conditions.

The available data on the progeny of carriers of 21~(21) type translocations are summarized in Table 5.

TABLE 5—*Progeny of Carriers of a 21~(21) Translocation*

| Carrier Parent | Children | | | | | |
|---|---|---|---|---|---|---|
| 21~22 (likely) | N.46 | N.45(T) | Tris.-21, 46(T) | Tris.-21* | N* | Total |
| Fathers: 2 | | | 4 (2) | 4 | | 8 |
| Fathers: (mosaics): 2 | | | 2 (1) | 1 | 4 | 7 |
| Mothers: 2 | | | 3 | 2 | | 5 |
| | | | 9 (3) | 7 | 4 | 20 |
| 21~21 (likely) | | | | | | |
| Fathers: 2 | 7 | 5 | 1 (1) | | | 13 |
| Mothers: 2 | | 3 | 1 (1) | | | 4 |

( ) No. indicates the number of propositi; (T) indicates carrier of translocation.

Compiled after the data of Hamerton et al. (1961); Forssman and Lehmann (1962); Fraccaro et al. (1960); Shaw (1962); Mukherjee et al. (1962); Dallaire et al. (1962).

* Only the phenotype is known.

As illustrated in Figure 6 the progeny of an isochromosome 21~21 carrier should be composed exclusively of 21-trisomics, since the haplo-21 zygotes are probably lethal. The families described by Forssman and Lehmann (1962) and Dallaire et al. (1962), seem to fit this prediction, being composed of 13 21-trisomics among 13 children. Conversely, the family reported by Shaw (1962), is in favor of a 21~22 translocation since she finds 2 21trisomics among 18 children with seven normal children and eight translocation carriers. Finally, the examples of mosaicism of Fraccaro et al. (1960) and Hamerton et al. (1961), can be attributed to either type, as the appearance of normal children is an insufficient reason to exclude the 21~21 type of translocation.

Although it has been reported that paternal age may act as a predisposing factor this has not yet been proved in cases of 21-trisomy caused by 21~21 translocation (Penrose, 1962). The only conclusion to be drawn from the available data is that contrarily to the 21~(13) type, the 21~21 translocation induce 21-trisomy as often at least in the progeny of male carriers than in the progeny of carrier mothers.

Before closing this brief discussion of the data it must be stressed that many of the translocation 21-trisomies are not transmitted, but arise *de novo* in the affected child (Gustavson, 1962; Carter, Hamerton, Polani,

TABLE 6—*Mosaics for 21-Trisomy*

| Authors | Tissue | No. of Cells | Percentage of Karyotypes | | | | Remarks |
|---|---|---|---|---|---|---|---|
| | | | Diplo-21 | Triplo-21 | Tetra-21 | Other | |
| Clarke et al. (1961) | Skin | 89 | 53% | 47% | | | ♀ 2½ yr.; morphology and dermatoglyphs are typical; I.Q. 100; no 21-trisomic cells in the blood |
| mother 26 yr. | | 44 | 38% | 62% | | | |
| father 26 yr. | Blood | 22 | 100% | 0% | | | |
| Fitzgerald and Lycette (1961) | Blood | 100 | 42% | 53% | 5% | | ♂ 51 yr.; low grade mental deficiency; diagnosis evident, few stigmata lacking (no epicanthus no simian crease, normal ears) |
| mother ? | | | | | | | |
| father ? | | | | | | | |
| Gustavson and Ek (1961) | Skin | 60 | 30% | 30% | | 40% | ♂ 12 yr.; had a brother 21-trisomic, dead at 6 months; low grade mental deficiency; typical deformities; trisomy-21 plus a small metacentric (21-21) (?) |
| mother 24 yr. | | | | | | | |
| father 29 yr. | | | | | | | |
| Nichols et al. (1962) | | | | | | | No details available |
| Warren et al. (1961) | Blood | 93 | 58% | 27% | ? | 15% | ♀ Typical aspect; annular pancreas; two other cases cited, one observed by Lytt, the other by Conen |
| Hayashi et al. (1962) | | 100 | 55% | 45% | | | ♂ 3 yr.; incomplete picture of 21-trisomy; I.Q. reported in the normal range |
| mother 17 yr. | Blood | 18 | 57% | 43% | | | |
| father ? | | | | | | | |
| Richard and Stewart (1962) | Blood | 45 | 48% | 52% | | | ♂ Typical case; low grade mental deficiency |
| Lindsten et al. (1962) | Blood and Skin | 293 | 62% | 35% | | 3% | ♀ Mild clinical picture although diagnosis is evident; borderline intelligence |
| Blank et al. (1962) | Blood | | | | | | ♀ Mother of a typical 21-trisomic; few stigmata of the disease |
| mother 40 yr. | | | | | | | |

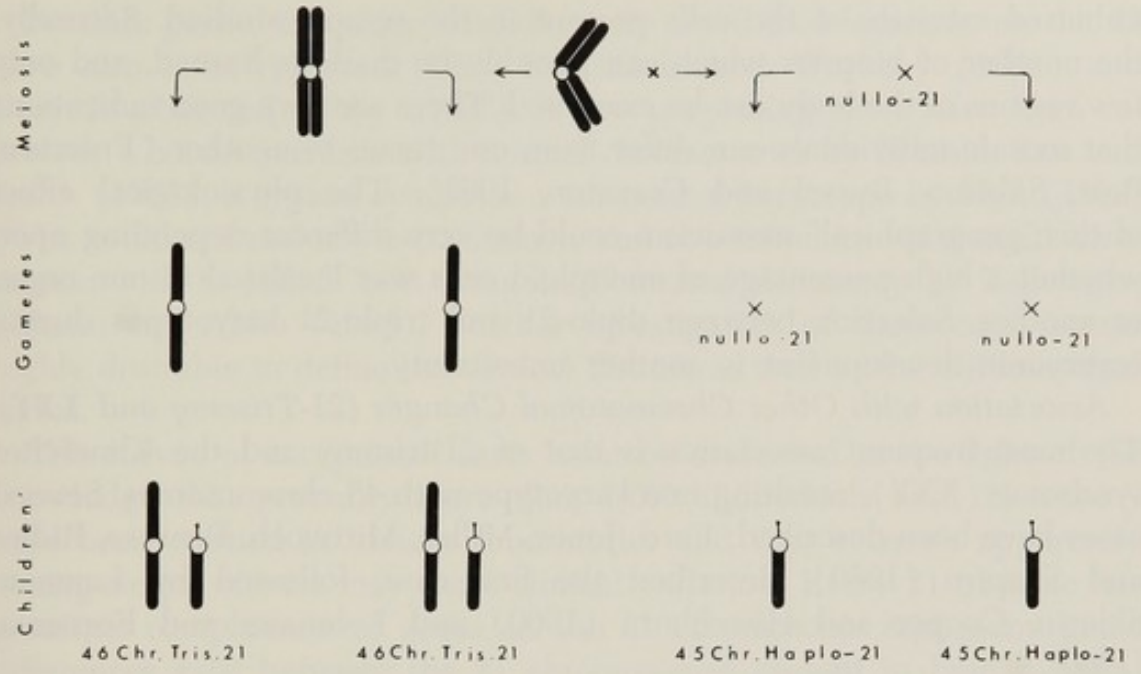


FIG. 6—*Progeny of a 21∼21 translocation carrier.* At meiosis the new chromosome 21∼21 has no homologue and moves directly to one pole, the other cell being entirely deprived of 21 chromosome. As seen the progeny can only include 21-trisomics or miscarriages.

Note: In case of a 21∼22 translocation, the transmission would be analogous to the case of a 21∼(13) translocation (Fig. 5).

Gunalp and Weller, 1960; Scherz, 1962). Both a 21-trisomic with 47 chromosomes and a 21-trisomic caused by translocation have been observed in a sibship of which both parents were normal (Penrose, 1963).

*Mosaic Trisomies.* Although 21-trisomics seem to be affected in every part of their economy, careful investigation has shown that some individuals, exhibiting the 21-trisomy syndrome in its classical form, or in a less clear cut picture ("mild affection") were, in part, mosaics. Their cell population was not uniformly composed of 21-trisomic cells, but included a varying proportion of normal, diplo-21 cells. The data are summarized in Table 6.

From these few data, it is not yet possible to deduce a relationship between the percentage of the 21-trisomic cells and the mental and physical status of the individual, although the patient with tetra-21 cells showed a particularly severe mental retardation.

Related to this problem is the biological relevance of the percentage observed. We have no good evidence that 21-trisomic cells and 21-diploid cells do not compete against each other under the conditions of observation. If this indeed is the case, the number of mitoses observed is not an

unbiased estimate of the cells present in the sample studied. Secondly, the number of biopsies which can possibly be made is limited, and only few regions of the body can be examined. There are very good indications that mosaic individuals can differ from one tissue to another (Fraccaro, Bott, Salzano, Russel and Cranston, 1962). The physiological effect of this "geographical" mosaicism could be very different depending upon whether a high percentage of aneuploid cells was localized in one organ or another. Selection between diplo-21 and triplo-21 karyotypes during embryonic development is another uncertainty.

*Association with Other Chromosomal Changes (21-Trisomy and XXY).* The most frequent association is that of 21-trisomy and the Klinefelter syndrome (XXY), resulting in a karyotype with 48 chromosomes. Several cases have been described; Ford, Jones, Miller, Mittwoch, Penrose, Ridler and Shapiro (1960), described the first case, followed by Lanman, Sklarin, Cooper and Hirschhorn (1960) and Lehmann and Forssman (1960).

A case of monozygotic twins, both 21-trisomics + XXY, was published in 1961 by Hustinx, Eberle, Geerts, ten Brink and Woltring and a similar case of twins has been recorded in our laboratory (Inst. Prog. obsv. No. 721).

The expected converse association between 21-trisomy and the Turner syndrome has not been reported. We have searched for 21-trisomic females exhibiting pterygium colli and found one such case but her karotype was 21-trisomy, diplo-X. It is possible that the severe genetic imbalance of the XO condition superimposed on the deleterious effect of the 21-trisomy does not allow the embyronic development of such a zygote.

Another association with 48 chromosomes, 21-trisomy and 18-trisomy, has been described (Gagnon, Katyk-Longtin, de Groot and Barbeau, 1961) but the malformed infant, exhibiting the physical stigmata of both syndromes, was not viable.

*Related Malformations.* Atypical cases, relating to chromosomal changes, have also been described. A girl exhibiting a congenital malformation looking somewhat like 21-trisomy but having probably a 21-16 translocation, has been reported by Böök, Gustavson and Santesson (1961). Also a mosaic boy exhibiting in some cells a very small extra chromosome has been recorded by Ilbery, Lee and Winn (1961), and is considered a "partial 21-trisomic."

Other cases showing trisomy for a small acrocentric have been quoted repeatedly. Firm conclusions on the genetic identity of the chromosomes involved cannot be drawn from a consideration of these cases (Lejeune, 1962).

Possible partial trisomies involving very small translocations on the 21 have also been reported: Chu, Rubinstein and Warkany (1961) and Gray, Mutton and Ashby (1962). A few perplexing cases have been reported: a borderline case with normal chromosomes by Schmid, Lee and Smith (1961); a typical syndrome with normal karyotype by Hall (1962) (who could not exclude mosacicism); and two trisomics for a small acrocentric with so-called "non-mongoloid" mental deficiency by Zellweger, Mikano and Abbo (1962). Further investigation of these cases seems highly desirable to define the clinical disease as well as the chromosomal status.

This survey of exceptional cases must be evaluated in the light of the knowledge that probably more than 1,000 individuals with 21-trisomy have been studied in various laboratories and that even an apparently homogeneous disease may show clinical variation. Every 21-trisomic has a unique complement of genes on the 21 chromosome and many genic differences exist between the 21 chromosomes present in human population.

In addition, as in the case of mental retardation observed by Ellis, Marshall and Penrose (1962), the extra chromosome can be rearranged, resulting in trisomy for various chromosomal segments.

*Pathogenesis of the Chromosomal Aberrations.* General factors predisposing to abnormal segregation of chromosomes have been recently reviewed (Lejeune, 1962) and only the data directly relevant to 21-trisomy will be discussed here.

*Characteristics of Chromosome 21.* The presence of satellites on this chromosome and the relation between these bodies and the nucleolus has led to the view that this type of acrocentric could be at greater risk of abnormal segregation than others. Mitotic association of acrocentric chromosomes is well substantiated (Ferguson-Smith and Handmaker, 1961). Thus, chromosome 21 could be at greater risk of translocation with another satellited acrocentric (Ohno, Trujillo, Kaplan and Kinosita, 1961). The existence of the (13)-trisomy (Patau, Smith, Therman, Inhorn and Wagner, 1960) and of the numerous 21~(13) and 21~21 translocations previously reported strongly supports this argument. Also the identical time of replication of these two types of chromosomes points in the same direction (Gilbert, Muldal, Lajtha and Rowley, 1962).

*The Interchromosomal Effect.* The evidence that structural changes of chromosomes increase the risk of abnormal segregation of the X chromosome has been amply demonstrated in Drosophila by the work of Sturtevant (1944). The same effect seems to be detectable in man.

The structural effect is clearly demonstrated in the progeny of carriers of a translocation involving the 21. Moreover, the translocation can in-

duce abnormal segregation of the 21, even if this chromosome is not transmitted to the trisomic child. This is well exemplified in the family described by Moorhead, Mellman and Wenar (1961), in which a mother carrying a probable 22~(13) translocation gave birth to a child with a free 21-trisomy, and without having received the translocated chromosome.

In a family described by Shaw (1962), the mother had an apparent deletion of a small arm and satellite of a 21 (or 22). She had five children; two were normal (both showing the "marker" chromosome), and three were 21-trisomics with 47 chromosomes (one of them exhibited the marker chromosome and the other did not). In a second family the mother of two typical 21-trisomics with 47 chromosomes had a normal set of 46 chromosomes but exhibited an excess of genetic material in one of the big acrocentrics. This excess, possibly including part of the 21, is thought to be the cause of the mild "stigmata" found in her phenotype. It seems likely that these chromosomal rearrangements make the meiotic pairing unstable or irregular and thus increase the risk of mis-segregation of homologues, or even of chromosomes which are not homologous with the rearranged one.

A less likely possibility is that chromosome rearrangements increase the risk of mis-segregation in the somatic cells. This is well known in Drosophila melanogaster in which gynandromorph mosaics result from the elimination of a ring-X chromosome (Brown and Hannah, 1952). Some data in man also point in this direction. Segmentary heterochromia of the iris, supposed to be due to chromosomal change when produced by irradiation (Lejeune, Turpin, Rethore and Mayer, 1960), has been recorded in a normal child belonging to one of the sibships described by Shaw and listed above (the mother with a possible 21~(13) translocation). Also this effect on meiotic and mitotic stability would possibly explain the accumulation of chromosomal diseases in certain sibships (Mosier, Scott and Cotter, 1961; Böök, Santesson and Zetterqvist, 1961; Hauschka, Hasson, Goldstein, Koepf and Sandberg, 1962; Cooper and Hirschhorn, 1961; Benirschke, Brownhill, Hoefnagel and Allen, 1962; Johnston, 1961; Zellweger and Mikano, 1961) and it seems at least an attractive hypothesis to suggest that familial recurrence of 21-trisomy could be explained by some undetected structural rearrangements in the chromosomes of parents.

Such an instability of the karyotype could be related to the occurrence in these families of a neoplastic process, such as leukemia (see below) (Buckton, Harnden, Baikie and Woods, 1961; Miller, Breg, Schmickel and Tretter, 1961; Hungerford, 1961).

*Aging of the Mother.* This predisposing factor, as previously mentioned, has no clear cut counterpart in the experimental animal; at least the data

are not available. Nevertheless in the case of somatic loss of the ring X-chromosome quoted above, the aging of the eggs has a very striking effect (Hannah, 1955). The same effect was found by Patterson, Brewster and Winchester (1932) on production of XXY, but only if the eggs were irradiated with x-rays.

The maternal effect, as far as experimental data are concerned, appears to affect somatic cells more than the meiotic process. This possible restriction to the blastomeric stage could be compatible with 21-trisomy, since there are instances in which the occurrence of the chromosomal disease is clearly postzygotic.

*The Time of Occurrence of the Aberration.* As discussed in the problem of translocations, there are instances in which we can assume fairly safely that a diplo-21 gamete was produced by a carrier parent. The same is true in the rare cases of reproduction of a 21-trisomic mother. Only in these instances which are obviously very rare (less than a few per cent of the total frequency of the disease) can we assume confidently that the error occurred at the meiotic process.

In contrast, two types of data show that the error can be postzygotic. The first concerns those cases of mosaicism in which obviously the "variant cells" were produced after gametic fusion. The probability of a double fertilization proposed by Gartler, Waxman and Giblett (1962) to explain their case of hermaphroditism can certainly not apply to all the mosaics with 21-trisomy. The second evidence is furnished by the exceptional type of twinning, the "heterokaryotic monozygotism" described by Lejeune, Lafourcade, Scharer, de Wolff, Salmon, Haines and Turpin (1962). A pair of monochorionic twins was observed who were identical for all the blood groups tested, but who differed in that 21-trisomy occurred in one while a normal karyotype (and phenotype) occurred in the other. A case of monozygotic twins differing in one chromosome had been previously observed (Turpin, Lejeune, Lafourcade, Chigot and Salmon, 1961; Lejeune and Turpin, 1961). This instance, an XO Turner and her XY normal monozygotic co-twin, is no longer unique, since a similar set of twins has been recently observed by Edwards, Dent and Crooke (1963, cited by Lindsten et al., 1963).

Figure 7 shows one possible way of producing these twins. Alternatively the twins could arise from a 21-trisomic egg and a secondary loss of the extra chromosome at the blasteromeric stage. In this second hypothesis the normal twin would have been naturally "cured" of his congenital disease.

If spontaneous mosaicism for the sex chromosome, as well as its production in animals by irradiating the fertilized egg (Russel and Saylors, 1961), is taken into consideration, the general picture emerges that the

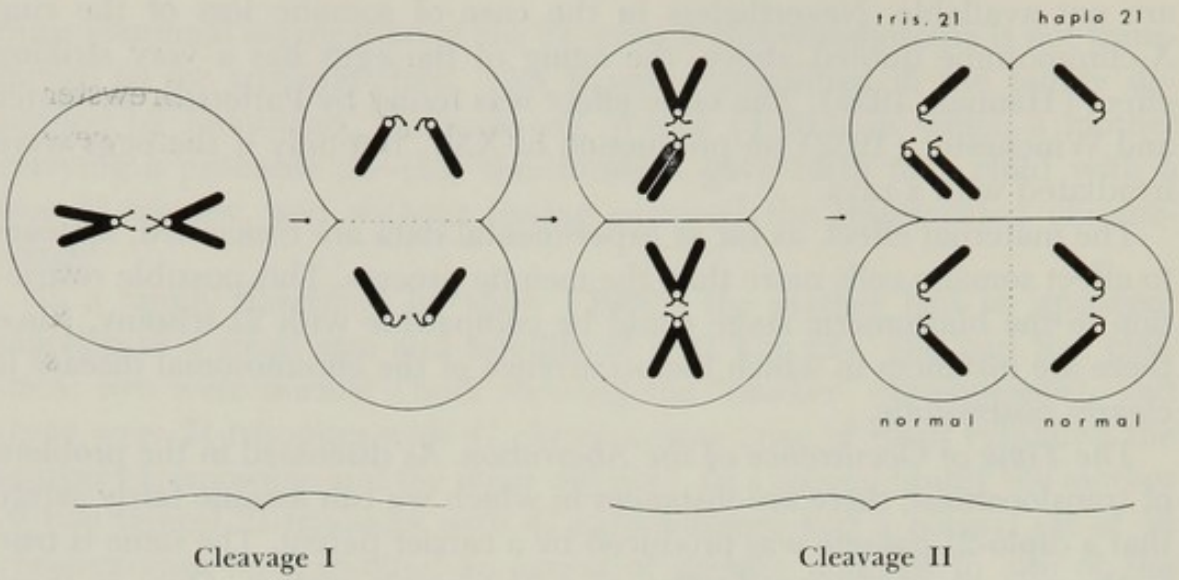


FIG. 7—*Abnormal segregation at the blastomeric stage.* Only the *21* chromosomes are represented. First mitosis of the zygote is normal. Then, one of the blastomeres divides normally, the other one give rise to two abnormal cells: a 21-trisomic and a Haplo-21 (which probably disappears). If cleavage in two embryos occurs at this four cell stage, the result is monozygotic heterokaryotic twins (see text). If only one embryo develops, the result will be a diplo 21/triplo 21 mosaic (see text).

most sensitive stages of development are the time of the fusion of the gametes or the first few cleavages of the blastomeres. If this hypothesis is true, it could very well be that most of the 21-trisomies are not of gametic origin, as generally accepted, but blastomeric, with the eventual death of the recripocal clone (haplo-21).

*The Leukemic Process.* The strong association between 21-trisomy, free or by translocation (German, DeMayo and Bearn, 1962), and acute leukemia is well known. Few karyotypic studies have led to precise results. Tough, Court Brown, Baikie, Buckton, Harnden, Jacobs, King and McBride (1961), reported five cases of apparently unchanged karyotype in the peripheral blood of 21-trisomic children affected by an acute leukemia. These authors raise the possibility that the true leukemic cells were not, in fact, recorded in the mitoses and that this negative finding is not definite.

Recently, leukoblastic abnormality was observed in a 21-trisomic girl in whom a chromosomal variation leading to an abnormal karyotype of 54 chromosomes was observed in the bone marrow and in the blood (Lejeune, Berger, Haines, Lafourcade, Vialatte, Satge and Turpin, 1963). The hemorrhagic disorder, which was characterized by purpura and bleeding, necessitated many blood transfusions, was diagnosed at birth and was still present at 2 years and 2 months. Analysis of cells revealed a

chromosomal complement which varied from 47 to 55 and showed that each number was correlated with a specific karyotype. The additional chromosomes were (in increasing number) small acrocentrics (v), big acrocentrics (T), medium sized metacentrics (M) and small metacentrics (c).

Two facts emerged from this study: (1) Every karyotype of an immediately greater number contained all the supernumeraries found in the karyotype of an immediately inferior number, plus one; (2) every new supernumerary occurred twice before the acquisition of a new one.

The general data are summarized in Table 7.

TABLE 7—*Association Between Karyotype and Chromosome Number in the Cells of a Trisomy-21 Patient with Leukemia*

| No. of Chromosomes | Karyotype | No. of Cells |
|---|---|---|
| 47 | tris.-21 | 22 |
| 48 | tris.-21+1v | 3 |
| 49 | tris.-21+2v | 1 |
| 50 | tris.-21+2v+1T | 1 |
| 51 | tris.-21+2v+2T | 1 |
| 52 | tris.-21+2v+2T+1M | 3 |
| 53 | tris.-21+2v+2T+2M | 7 |
| 54 | tris.-21+2v+2T+2M+1c | 36 |
| 55 | tris.-21+2v+2T+2M+2c | 3 |
| 62 | tris.-21+2v+2T+2M+2c+18+5M+2 | 1 |
| | Total | 78 |

Exceptions: One cell with 51 chromosomes : tris.-21 + 2v + 1T + 1M; one cell with 53 chromosomes : tris.-21 + 2v + 2T + 1M + 1c. The symbols used, described in Lejeune (1960), represent: v = group (21-22); T = group (13-15); M = group (6-12-X); c = group (19-20).

Although this apparent evolution of a clone is not yet substantiated by other observations, it is worth noting that a previous observation of a 47/49 mosaic was reported in the bone marrow of a 21-trisomic girl by Piazzi and Rondinini (1961) (it is not known if this child was suffering from leukemia). The observation by Ross and Atkins (1962) of another 21-trisomic with leukemia also showed 47/49 mosaicism in the blood. In both these instances the 49 chromosome karyotype was due to a typical 21-trisomy with two additional small acrocentrics (21-22).

The availability of only three cases for study prevents any generalization, but it is tempting to suppose that is was not by chance in the three

instances now known that the karyotypic variations started with the same type of chromosome and with the appearance of two additional identical ones.

Other examples of the presence in duplicate of additional chromosomes in neoplastic disease have been reported. One case concerns apparently normal big acrocentrics (13-15) (Sasaki, 1961), and another obviously rearranged giant acrocentrics in cells of exudate of cancerous origin (de Grouchy, Vallee and Lamy, 1963).

The possibility of chromosomal interaction resulting from the trisomy in the causation of leukemic process in the 21-trisomic children is for the moment an open question, as also are the possible laws controlling the karyotypic changes in these neoplastic growths.

*The Genic Content of the 21 Chromosome.* The study of chromosomal duplication (or deletion) as a means of detecting genes which show a dosage effect has been a very powerful tool in experimental cytogenetics. Its application to the mapping of human chromosomes has been attempted, especially in the 21-trisomy syndrome. In such research very great care has to be exercised in the selection of phenotypic characteristics as "markers." Fine and immutable morphological traits, like dermatoglyphs, have been proposed, but their alteration in all three different types of trisomies (see above) prevent any conclusion about the precise location of the hypothetical genes controlling them.

Comparisons between members of a monozygotic heterokaryotic set of twins for somatic traits are also possible, since these individuals are genetically identical except for one 21 chromosome. In the case quoted above, the 21-trisomic differed from his normal co-twin by having straight hair, whereas the normal boy has curly hair. It cannot be concluded that the "straight hair gene" is on chromosome 21, but only that some genes on this chromosome interfere with the manifestation of this character.

The difficulty of genetic analysis with morphological traits is well known. Biochemical characteristics seem more satisfactory as they are probably nearer to the primary products of gene activity.

*Alkaline Phosphatase Activity of Polymorphic Leukocytes.* The estimation of alakaline phosphatase activity in polymorphic leukocytes provides an apparently simple biochemical test applicable at the cellular level. Since the first report of Alter, Lee, Pourfar and Dobkin (1962), numerous publications have confirmed the fact that alkaline phosphatase activity is higher in 21-trisomics than in normal children. The ratio of activities is of the order of 3 to 2, and is strikingly similar to the ratio of 21 chromosome numbers (Trubowitz, Kirman and Masek, 1962; King, Gillis and Baikie, 1962). No correlation is found with the Arneth count (Lennox, White and Campbell, 1962), or with the neutrophil count (King, Gillis and Baikie,

1962). Thus the relation between alkaline phosphatase activity and the karyotype seems independent of the tendency of 21-trisomics to incur infections. The tentative localization of the structural gene on the 21 chromosome is nevertheless rendered insecure by the negative correlation between this enzymatic activity and prepubertal age, which suggests that a correlation may exist with the level of sex hormones (Alter, Dobkin, Pourfar and Lee, 1963). Nevertheless the conservative hypothesis that genes affecting alkaline phosphatase activity are located on the 21 can reasonably be made, with the reservation that other genes, possibly many, can also influence this trait.

Another indication that alkaline phosphatase activity may be related to the genes on chromosome 21 arises from the observation of a deleted small acrocentric ($Ph^1$ chromosome) in leukocytes of patients suffering from chronic granulocytic leukemia (Nowell and Hungerford, 1960; Tough, Court Brown, Baikie, Buckton, Harnden, Jacobs and Williams, 1962). If the deleted chromosome is indeed the 21, the low phosphatase activity found in this disease (Valentine and Beck, 1951) could be attributed to the loss of the locus involved.

In addition the observation of haplo-(21 or 22) clones in acute myeloblastic leukemia (Ruffie and Lejeune, 1962) supports the supposition, drawn from the $Ph^1$ chromosome, that genes exist on the 21 which are concerned with the regulation of polymorphonuclear production.

It must be stressed that this second type of inference is based on the assumption that the $Ph^1$ chromosome is really a deleted 21. This tempting assumption, although not proved, could also account for the shift to the left of the low Arneth count in 21-trisomics. By this hypothesis a simple mechanism could be postulated: a balanced karyotype gives normal counts, a triplo-21 depresses the lobulation of the nuclei of the polymorphs and a partial or total deletion of the 21 leads to an excessive proliferation of this type of cell.

As has been shown in the paragraph concerning leukemias in 21-trisomics, an extra supernumerary of this same acrocentric type can also be encountered in the leucoblastic reaction. The significance of this last effect is not immediately apparent, but we can at least imagine that hyperrepression of the granulocytic differentiation could result in blastic proliferation.

*Biochemical Changes.* Biochemical changes may be more sensitive indicators of gene overdosage than cellular effects. Many biochemical traits have been reported to be peculiar in 21-trisomics. Thus blood calcium level (Stern and Lewis, 1958) and serum esterase level (Stern and Lewis, 1962) are reported to be lower in 21-trisomics than in normal individuals, whereas the serum uric levels (Fuller, Luce and Mertz, 1962) are

reported to be higher. In a systematic search for biochemical changes related to 21-trisomy, tryptophan metabolism has been investigated, and a decreased urinary excretion of xanthurenic acid and of 5-hydroxyindolacetic acid has been reported (Gershoff, Hegsted and Trulson, 1958; Gershoff, Mayer and Kulcyzcki, 1959; O'Brien, Groshek and Streamer, 1960). As observed by Jerome, Lejeune, and Turpin (1960) and Jerome (1962), a decreased excretion of 5-hydroxyindolacetic acid, xanthurenic acid, kynurenic acid, and indolacetic acid seems to be a biochemical symptom of the disease. No direct relation between these metabolic changes and chromosomal constitution has yet been established, although acceleration of some steps by enzymatic overdosage has been discussed. This hypothesis of over production of enzymes by trisomics seems to be confirmed by the alkaline phosphatase data, but cannot yet be related to experiments in other species. In bacteria, however, there are indications that genetic overdosage induces an excess of enzymatic activity.

## Conclusions

Although chromosomal research has greatly improved our knowledge of the disease, the pathogenesis of 21-trisomy has still to be discovered.

Two main questions need to be answered:

1. How can normal genetic information be deleterious if in excess?
2. What biochemical changes are produced by this imbalance of the karyotype?

The cytogenetic epoch of 21-trisomy has obviously not closed, but it is hoped that a biochemical era will soon open. The discovery of specific metabolic changes in 21-trisomics would be an outstanding advance, and the successful control of these changes, if realizable, would receive a most precious reward: the first reasonable hope of correcting the catastrophic consequences of an inborn chromosomal error.

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Chapter 6

# Multifactorial Inheritance and Human Disease

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The concept of multifactorial inheritance is almost as old as the subject of genetics itself, and was, of course, invoked to explain the inheritance of continuously distributed metrical characters. Methods of analysis of great refinement and power have been developed in connection with experimental studies on animals and plants. With man, however, this is one of the fields where the necessary reliance on observation instead of experiment, and also the length of generations, are severe handicaps. Only a fraction of the information which can be elicited in experimental forms is available in man. In man we are restricted to a few things, such as the form of frequency distributions, and measures of the resemblances between relatives of different degrees, and these, for obvious practical reasons, can usually be close relatives only. Nevertheless, even such partial knowledge as can be attained may be of much practical importance.

In recent times the term polygenic has been widely used, although in two ways. Some writers, for example, Stern (1960), treat the terms polygenic and multifactorial as synonymous and apply them to any instance in which the inheritance of an observed character is determined by the combined action of more than one gene pair. These may be as few as two. Others, for example Darlington and Mather (1949), describe inheritance as polymeric if the action of gene pairs is similar and supplementary, but require a third criterion before describing it as polygenic. This is that the

effect of any single gene shall be small in relation to the total variation. Thus all polygenic inheritance is polymeric, but not all polymeric inheritance is polygenic. The distinction is chiefly a practical one. If only a very few gene pairs are concerned, or if one gene pair exercises an influence which is large in comparison with the total variation, then in experimental forms (although hardly in man) the methods of classical Mendelian analysis can be used. With polygenic inheritance in the narrower sense quite different methods of biometrical analysis are needed. In practice, of course, instances of inheritance which are polymeric without being polygenic are rare in comparison with multifactorial inheritance of the more usual kind.

It should hardly be necessary to emphasize that it is not supposed that the relatively small effects of genes making up polygenic systems need be their only effects. It has been known for 50 years that ordinary major genes, whose principal action is to produce a recognizable qualitative difference, may have ancillary effects on many metrical characters. Many major genes known in man have small average effects on such characters as stature. Thus major genes play their part in polygenic systems. In a word, major genes may have additional minor effects; but it is evident that the converse is not true. There are minor genes which produce no major effect. It is difficult to define the extent to which major genes play a part in determining polygenic variation. Some might hold that it could be considerable; others, that it is probably small in most instances in comparison with the total hereditary variation. A human example of the contribution of two known major gene loci in contributing to hereditary likeness is given later in this chapter.

## The Theoretical Basis

The basis for the genetic treatment of continuous variation was provided by Fisher (1918) in a classical paper. He showed that not only were biometrical observations compatible with Mendelian theory, but that, in fact, they could not be rationally explained in any other way. The theoretical measures of resemblance between relatives, given certain simplified assumptions, turn out to be very straightforward. In terms of regression coefficients, and usually of correlation coefficients also, they are equal to the number of genes in common. The expression "genes in common," in this context, means the proportion of identical genes derived from the same ancestor genes. The remainder of the genes may be the same or different, depending on their frequencies in the population. In regard to these other genes relatives resemble each other to the same extent as do,

on the average, unrelated persons. The proportion of genes in common are shown in Table 1 for a number of relationships. These figures also give the measures of resemblance in terms of regressions. As the two regressions are equal, they are also the correlations coefficients.

TABLE 1—*Genes in Common*

| Relationship to a Given Subject | Proportion of Genes in Common |
|---|---|
| Identical twin | 1 |
| Parent, child, sib, fraternal twin | 1/2 |
| Grandparent, grandchild, uncle, aunt, nephew, niece, half-sib, double first cousin | 1/4 |
| First cousin | 1/8 |
| First cousin once removed | 1/16 |
| Second cousin | 1/32 |
| Third cousin | 1/128 |

The simplified assumptions under which the theoretical measures of resemblance are realized are: (1) that inheritance alone is involved; (2) that the gene pairs do not show dominance and recessiveness, that is, that the heterozygote is intermediate between the two homozygotes; (3) that there is no assortative mating. It is especially important to note that the correlations are the same as those for a single gene pair without dominance.

Assortative mating increases the measures of resemblance, but can be allowed for to a certain extent. In any event, with human metrical characters, apart from intelligence, assortative mating tends to be moderate in amount.

Dominance needs special treatment. On the simplified model, without dominance, the frequencies of the alternative alleles in the population are immaterial, but with dominance, the correlations depend on gene frequency. Thus, with complete dominance, if 'a' is the frequency of the recessive allele, the parent-child correlation becomes

$$\frac{a}{1+a}$$

and the sib-sib correlation

$$\frac{1+3a}{4(1+a)}$$

The results for a single gene are illustrated in Figure 1. In the limit, when the dominant allele is extremely rare, the values of the regressions

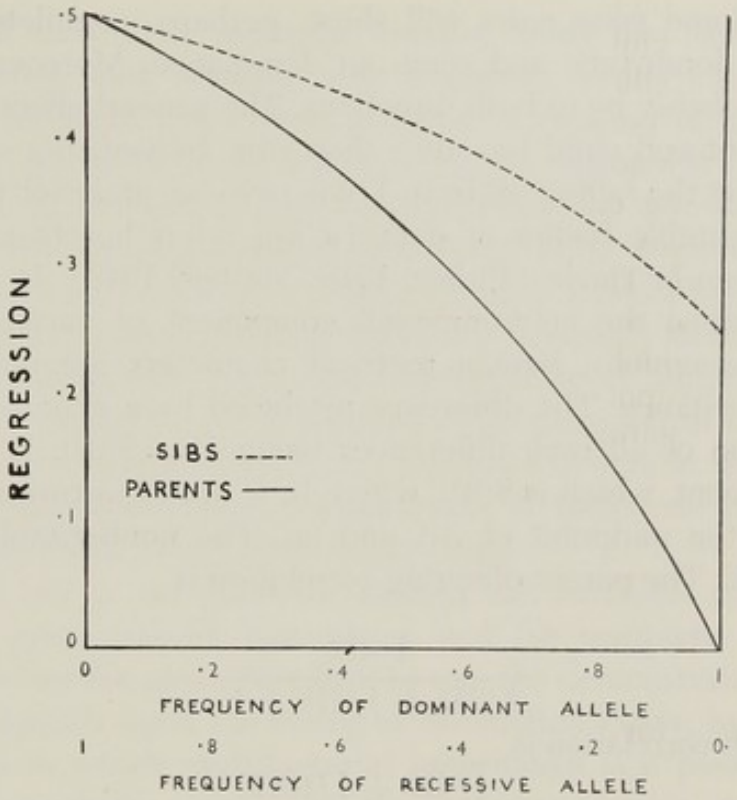


FIG. 1—Effect of dominance on the regressions of sibs and of parents on subjects (from Roberts, 1959).

are the same as for genes in common, namely, 1/2 for parent or sib, 1/4 for uncle and aunt, etc. At the other extreme, when it is the recessive allele which is exceedingly rare the regressions tend in the limit to zero for parent or child (and other relatives) and 1/2 for sibs. This can easily be seen without mathematical treatment. With a rare abnormal gene, if this is dominant, half the children are like the affected parent, half the sibs like the affected sib. But if the rare abnormal gene is recessive, exhaustive enquiries may fail to disclose a single affected person outside the particular sibship in question. In terms of what we can see, the correlation in regard to relatives other than sibs has fallen to zero; although not, of course, in terms of genes in common, only that the real resemblance is hidden by dominance. But however rare the gene, on the average a quarter of the sibs are affected in the same way as the subject.

An important point to note is that for any frequency of the alleles the reduction in the regression is twice as great for parents or offspring as it is for sibs. Thus, with an equal population frequency of dominant and recessive alleles the regression is 1/3 for parents and 5/12 for sibs; that is, the regression has fallen from the theoretical value, without dominance, of 1/2 by 2/12 for parents and 1/12 for sibs.

With a multifactorial system we can think of an averaged measure of

dominance. Some gene pairs will show, perhaps, complete dominance, some partial dominance and some no dominance. Moreover, the dominance will probably be in both directions. The general effect is, as before, to make parent and child less alike than sibs. Indeed, the same relationship holds, that the falling off from 1/2 is twice as great for parents as for sibs. Given suitable bodies of data (a big if) it has been shown that calculations can be made (Fisher, 1918; Mather, 1949). It is also necessary to introduce the environmental component of variation, for with hardly any exceptions, human metrical characters are not determined solely by inheritance. The difference produced by a gene pair is defined as 2d, the sum of all such differences being $D = S(d^2)$. H is the dominance component, which is $S(h^2)$, where $h_a$ is the departure of the heterozygote from the midpoint of *AA* and *aa*. The nonheritable component is defined as E. The parent-offspring correlation is

$$\frac{1/4D}{1/2D + 1/4H + E}$$

and the sib-sib correlation is

$$\frac{1/4D + 1/16H}{1/2D + 1/4H + E}$$

If data are available for estimating the two correlations, the relative magnitude of D, H and E can be determined.

## The Practical Difficulties

As previously mentioned, this is one of the fields in which the limitations of human material are especially apparent. Most important of all, there is the necessary reliance on observation instead of experiment. Next, there is the length of the human generation, which confines direct observation to three or four generations at most. There is also the practical difficulty of extending direct observations to more distant relatives. Clearly it is very important to secure an unbiased sample, and with human material this usually means a complete sample; for apart from a few obvious limitations, such as a radius of distance, any loss may well be biased. Starting with a given sample of subjects, it is not a very difficult task to make contact with close relatives, namely, parents, sibs and children, that is, relatives of the first degree. When the subjects are relatively young, grandparents too may be included without too much difficulty; but, of course, a number will be unavailable through death, and this itself may mean a biased loss. It is when more distant relatives are sought

that practical difficulties multiply. Uncles, aunts and cousins may be widely scattered; and, starting with a disease which has brought the subject to a hospital and so has involved a measure of gratitude, this may well not spread to more distant relations. Some will consent to examination, others will not, so leading to possible bias one way or another; it is often difficult to guess which. It has also to be remembered that in conditions in which multifactorial inheritance is a probability, or a possibility, direct examination and measurements of various kinds may be necessary. This is different from definite deformities or abnormalities, with which hearsay evidence may, on occasion, be accepted as sufficiently reliable for practical purposes. The consequence is that in general we are restricted to measurements of, or measures of incidence in, quite close relatives, often those of the first degree only.

As pointed out in the previous section, the measures of resemblance for a single gene without dominance and for complete multifactorial determination are the same. Needless to say, the demonstration of a single gene effect depends upon the fitting of Mendelian ratios; but with nearly all conditions in which multifactorial inheritance is a possibility, determination is not wholly genetic and the numbers of relatives affected fall below Mendelian expectation. There is no longer any firm basis for fitting. We observe certain regressions or correlations, or certain incidences amongst relatives and these can in theory be equally well explained by a single gene showing reduced penetrance, or on the basis of multifactorial inheritance. With dominance, whole or partial, the only difference is that the resemblance between sibs is closer than that between parent and child. This may point to a recessive gene of partial penetrance, or to multifactorial inheritance with an average measure of dominance. Moreover, it always has to be remembered that when environmental influences are potent, and they usually are, sibs may tend to share a more similar relevant environment than do parent and child, and this can easily be misinterpreted as dominance.

It is difficult to devise any test which will distinguish unequivocally between the single gene and multifactorial inheritance as the explanation of an obvious genetic element in common diseases. Often, after reviewing the evidence, it becomes necessary to think of prior probabilities in the light of what is known in experimental forms, not a very satisfactory procedure, but the best available.

It may be noted in passing that twin studies do not help. What is observed is a measure of concordance between identical twins which is greater than that between fraternal twins. Again, this can be interpreted as the effect of a single gene of reduced penetrance or as the result of multifactorial inheritance. Furthermore, twin material is usually limited

in amount so that the measures of resemblance have wide margins of error.

In relation to human traits we can think of a descending order of probability. With certain continuously distributed normal measurements, a single gene effect is almost unthinkable, and biometrical methods appropriate to such variation can be confidently applied. No one would attempt to explain the genetics of finger print ridge counts, or stature, in terms of single gene effects (although, of course, single genes may occasionally have major consequences). Very few diseases, however, can be equated to continuously distributed measurements. Two which come near it, which are dealt with in subsequent sections, are high-grade mental deficiency and essential hypertension. With some other diseases there are indications of continuously distributed variation, not perhaps in regard to the disease itself, but in some of its concomitants.

Most human diseases, however, are of an all-or-none kind. Here, where there are clear indications of a genetic element in causation, multifactorial inheritance as an explanation of that genetic element is by no means ruled out. This would probably be accepted without question as inherently likely with genetic differences in resistance and susceptibility to some infectious diseases; it would fit in with studies on experimental animals. This is meristic, or quasi-continuous, variation, the underlying genetic degrees of predisposition being thought of as continuously distributed, although whether or not the individual contracts the disease is all-or-none, so that the variation in what we observe is discontinuous. Later in this chapter it is shown that analysis is not impossible, and that certain congenital malformations, which clearly have some genetic basis, do provide indications that the genetic basis is probably multifactorial. For the rest, it may well be that improved laboratory and other procedures will increasingly reveal the nature of the genetic predisposition, not, that is, the disease itself, but the genetic background which facilitates or hinders its appearance.

## An Example of Multifactorial Inheritance in Man: Fingerprint Ridge Counts

Before passing on to human disease it may be useful to consider briefly a most elegant example in connection with a normal metrical trait. This is provided by a series of studies by Holt on fingerprint ridge counts. Convenient summaries and bibliographies have recently appeared (Holt, 1961a and 1961b). The ridges are counted according to defined rules and the total for all the 10 figures is the variable considered. A great

advantage of this measurement is that the number of ridges is finally determined by the fourth month of fetal life, so there are no troublesome allowances to be made for differences in age, as is almost universally necessary with other measurmeents. There is a sex difference, but allowance for this makes practically no difference to the calculations. Table 2 shows the correlations between relatives, compared with the simplified theoretical values.

TABLE 2—*Fingerprint Ridge Counts: Correlations Between Relatives*
(From Holt, 1961b)

| Relationship | No. | Correlation | |
|---|---|---|---|
| | | Observed | Expected |
| Mother-child | 405 | 0.48 ± 0.04 | 0.5 |
| Father-child | 405 | 0.49 ± 0.04 | 0.5 |
| Midparent-child | 405 | 0.66 ± 0.03 | 0.71 |
| Sib-sib | 642 | 0.50 ± 0.04 | 0.5 |
| Identical twins | 80 | 0.95 ± 0.01 | 1.0 |
| Fraternal twins | 92 | 0.49 ± 0.08 | 0.5 |

It will be seen how closely the theoretical values are realized, the only significant difference being the slightly lower value for identical twins. There is no significant indication of assortative mating. It can be concluded, therefore, that this measurement is almost wholly determined by inheritance. The similarity between the parent-child and sib-sib correlations does not suggest any dominance effects. These can be examined further by an ingenious method devised by Penrose (1949). This makes use of the regression of child on midparent, that is, the mean of the parental measurements. The theoretical regression of child on midparent is 1, for all the child's genes come from the father and mother. The regression of midparent on child is, however, one-half, because only half the midparent genes are identical with those in the child, the others being on the average those of the general population. The regressions are thus asymmetrical, and the correlation coefficient is $\sqrt{(1 \times \frac{1}{2})} = 0.71$. This is in good agreement with the observed value shown in Table 2. Penrose points out that not only would dominance lower the regression of child on midparent, but that the regression would also become nonlinear. There is no sign of this in Holt's data, so it can be concluded that dominance and recessiveness do not play any appreciable part in determining the variation of total ridge count.

It is true that the frequency distribution of total ridge count shows some departure from the Gaussian curve, for there appear to be concen-

trations of observations at a few particular points. This may perhaps be due to a few gene pairs which are exercising an influence which is considerable in relation to the total variation. Nevertheless, this example remains a most satisfactory demonstration of the behavior of a metrical character in man which displays almost complete hereditary determination.

## Stature

Fingerprint ridge counts must be fairly unique amongst human metrical measurements in that the influence of environment is negligible, and also, perhaps, in that the dominance component is also negligible. Hence the theoretical correlations on the assumption of additive, intermediate pairs is very closely realized. With nearly all other measurements, however, environmental influences are contributing to the observed variation, sometimes to a small extent, often substantially, and sometimes to an extent far greater than genes. Stature provides an example in which the environmental component is relatively small. It also serves to introduce the concept of disease as related to a measurement. It must be remembered, however, that the relative influence of heredity and environment may differ in different environments. Thus, with stature, the statement that the environmental component is relatively small would be true only of a community in which gross malnutrition was rare, so that the great majority of individuals are able to reach something like their full genetic potential.

That the environmental component in variation is small is amply demonstrated by resemblances between relatives, and also by twin studies. But hereditary determination is no longer complete, and so we no longer have the yardstick of theoretical correlations.

Fisher (1918) made calculations on the data of Pearson and Lee (1903) on stature in parents and children, and in sibs. (See also Mather, 1949). Using the formulas given on page 182 for the relative magnitudes of D, H and E, representing, respectively, the hereditary, dominance and environmental components, he showed that Pearson and Lee's parent-offspring correlation of 0.4180 and sib-sib correlations of 0.4619 gave relative magnitudes as follows:

$$H = 0.4201D$$
$$E = -0.0069D$$

The nonheritable part of the variation is thus very small, becoming negative owing to sampling error. Taking E as zero, the variation due to domi-

nance is 17 per cent, the remaining 83 per cent being due to heritable factors (although some part of the dominance effects is included in this figure). With stature, there is an appreciable correlation between husbands and wives. Allowing for this, Fisher estimated that the contributions of H and D were approximately in the ratio 1:3.

The application of the formulas is interesting, but it must be admitted that their applicability to actual human data is distinctly limited. Relatively enormous numbers are required, and even so the sampling errors of the estimates are very large. Moreover, as they are ratios the fiducial limits are asymmetrical, so that large positive values will occur as often as small negative ones. There is a further point. With some measurements it may well be that sibs tend to share the relevant environmental factors to a greater extent than do parent and child. If so, the latter correlation is diminished to a greater extent than the former. This will mimic or inflate the dominance effect. An example showing the failure of the formulas to yield useful results is given in the subsequent section on arterial pressures.

Stature illustrates two points in the relationship of a metrical character to disease. The first is that although the frequency distribution is very fairly Gaussian over the great bulk of the range (see, for example, Martin, 1949), there are irregularities at the extremes. At the negative tail of the distribution there is an excess of very low statures, due to the inclusion of dwarfs of various kinds, such as achondroplastic dwarfs, rachitic dwarfs, renal dwarfs, midgets, some girls with Turner's syndrome, and others. Moreover, these low values, due to pathological causes, may overlap those due to persons whose short stature is to be attributed to multifactorial inheritance; in fact, the tallest achondroplastic dwarfs are taller than the shortest normal people. At the other end of the scale there is a smaller proportion of pathological giants. Interestingly, the Scottish Mental Survey of 1947 (Scottish Council for Research in Education, 1953, page 29) shows that when large numbers of subjects are measured there is a rather striking excess of very tall persons, who cannot be accounted for on the basis of a known pathology. Out of 7,000 children measured, seven were 6 feet tall or more at age 11 years (*i.e.*, 1/1,000). This frequency is more than 6 standard deviations above the mean. The expectation of this being due to chance, on the basis of a normal distribution, is less than one in 1,000 millions. No associated abnormalities were noted. The second point is that disease processes may produce diminution of stature, while still leaving the affected person well within the normal range. This is shown, for example, by some of the size differences between identical twins. The combined multifactorial-environmental system that controls variation in stature includes some minor effects of major

genes and also some definite accidents of development or disease processes affecting development. The correlations, however, and the form of the frequency distributions, indicate that the role of such factors must be relatively small in comparison with the additive effect of polygenes and of minor environmental influences.

## General Intelligence and Mental Deficiency

General intelligence, as measured by intelligence tests, is a graded character. The frequency distributions are reasonably normal. The heritable component is relatively high. The correlation between sibs is universally found to be of the order of 0.5 or slightly more. This by no means indicates something like complete hereditary determination, however. First, the correlations are appreciably raised by assortative mating, which is very strong. On the average, in communities like those of Western Europe or North America (where most of the observations have been made), husbands and wives tend to resemble each other in intelligence, because of deliberate choice, to a greater extent if anything than do sibs. The similar environment of sibs must undoubtedly raise the correlation between them to some extent; to mention one influence only, there is the background of culture in the home, and this does affect test performance. Nevertheless, the data on sibs, on twins, with studies on twins reared apart, and on foster children, do permit the drawing of some conclusions. It seems likely that something between a half and three-quarters of the variation is to be ascribed to heredity.

Probably no one seriously doubts that the bulk at least of the heritable variation is multifactorial. It is when we turn to mental deficiency that the picture becomes less obvious and more complicated. As we pass, then, from stature and dwarfism, or gigantism, to general intelligence and mental deficiency, we come to a measurement which itself in a sense defines an appreciable proportion of abnormality. It is difficult to draw a line, but in most communities perhaps about 3 per cent of the population must be judged on the usual criteria to be mentally defective.

The early Mendelians tried to make mental deficiency a character determined by a single recessive gene. This naive attempt, understandable in its day, was, of course, doomed to failure. Mental deficiency is not one thing, but many. An imperfectly developed brain, or one which for some reason functions imperfectly, is no more likely to represent an entity than the faulty development or functioning of any other bodily system.

The work of Binet and Simon (1907) revolutionized the study of mental deficiency by providing a metrical scale. It is worth recalling that at the

lower levels at least, scales like the Binet are not arbitrary. About 50 per cent of, say, 7-year children pass the tests at that year level. Some will do so at 5 years or at 6, others not until they are 8 or 9 years. But providing they are not mentally defective, all children will ultimately pass the 7-year level of tests. Thus the scale may be regarded as anchored to the biological reality of rate of development, which is itself correlated with ultimate level of development. It is true that at levels higher than those attained by the average child of 14 years, extrapolation must be employed, and mental ages become arbitrary. This, in fact, is probably what has led to a measure of unsatisfactoriness in the higher ranges of the Terman-Merrill revision, older very clever children being able to secure extremely high scores which do not fit a Gaussian distribution. In the dull and mentally defective part of the range, however, this difficulty does not arise.

One of the earliest psychologists to carry out a quantitative study was Jaederholm. He tested practically all the children of Stockholm who were in "help classes." The number tested was 301. He also tested a sample from the general population of schoolchildren for comparison. The results were analyzed in collaboration with Karl Pearson (Pearson and Jaederholm, 1914). The children in the help classes were practically all what we should now call feeble-minded; hardly any idiots and imbeciles were included. The I.Q. as a device for allowing for differences in chronological age had not then been invented, but inspection of the tables shows that only three of the 301 tested children had I.Q.'s below 50, the lowest being 45. What Pearson and Jaederholm showed was that test scores were continuously distributed and gave a good approximation to a Gaussian curve. They also showed that the children in the help classes formed part of this distribution; the fit was three times better if they were included, proportionately to their numbers in the population, than if they were excluded. Pearson and Jaderholm stated that it was clear that very low grade defectives, idiots and imbeciles, could not be accommodated in the normal curve. They also found some excess of low values above this level. They suggested that the causation of defect was pathological with the very defective children, whereas most of those in the help classes represented the negative tail of the Gaussian curve.

Pearson and Jaederholm's work was largely overlooked. Perhaps the break due to World War I had something to do with this. There was also the polemical and combative way in which the paper was written. It appeared in Pearson's most controversial series "Questions of the Day and of the Fray," in which opponents were, in Cromwellian fashion, sternly invited to "bite the dust"; it could fairly be concluded that this sudden interest in mental deficiency was due less to a desire to elucidate its causes than to finding a convenient stick with which to beat the Mendelians.

And, of course, the paper was ahead of its time; not until Fisher's synthesis of 1918, indeed, not until considerably later, were the arguments likely to appeal in the context of current Mendelian thought.

A subsequent attempt at dichotomy was due to Lewis (1929, 1933). As the result of his admirable large scale survey he drew a distinction between "sub-cultural" defectives, to be regarded as "normal deviants," and "pathological" defectives, to be regarded as "abnormal variants." Broadly, the first type could be equated to the feeble-minded, the second to the idiots and imbeciles. Subsequent experience has shown, however, that by no means all low-grade defectives are distinguished, to quote Lewis' words, by "some definite lesion or abnormality," nor that the sub-cultural group includes, on the other hand, those cases of mental defect "in which no such alien factor is found." It is true that definite entities have been identified, but they account for only a small proportion of mental deficiency, and the most useful general analysis so far has been in terms of the metrical character, I.Q.

Mental deficiency and I.Q. cannot be equated, but they are closely associated. Probably all those with an I.Q. below 45 will require institutional care, or its equivalent in the home. Between I.Q. 45 and, say, 65, the majority will do so, although the proportion diminishes as we pass up the scale. Above, say, 65 or 70 the proportion becomes small. Other factors are, of course, important, in particular emotional stability, and it is these factors which, at the higher levels, determine whether the individual needs care and control for his own protection, or for that of others. Incidentally, it may be questioned whether mental deficiency in the higher grades is primarily a medical problem; it is more a problem of educational psychology and social adaptation.

This overlapping has its counterpart in terms of causation. Many of those known causes which usually produce low-grade deficiency may on occasion produce no more than moderate retardation. With phenylketonuria, due to a recessive gene, the usual result is severe defect, but a few subjects are no more than feeble-minded, and indeed may extremely rarely fall within normal limits of intelligence. The high grade sufferer from Down's syndrome (mongolism) is rare, but he exists. Thus, at the outset it has to be recognized that some of those presumed to owe their deficiency to multifactorial inheritance (to the extent that heredity is involved) will have lower I.Q.'s than a number of those whose deficiency is to be ascribed to a single abnormal gene or to some particular accident of development. It must also be recalled that errors of measurement will also contribute to overlapping; the mean difference in I.Q. on retesting is about 4 points.

A more detailed examination of the negative tail of the frequency curve

was attempted by Roberts, Norman and Griffiths (1938), and summarized by Roberts (1950). A practically complete sample of 3,400 children was tested on a group scale and the 23 per cent of children securing the lowest scores were also retested. The close correlation between the scores on the two scales (0.89) enabled the mean on the Binet scale to be estimated with the same accuracy as though a random sample of 710 individuals had been tested, and the variance as though 495 children had been tested. The negative tail was, of course, represented in full. The results are shown in Table 3. It will be seen that the fit is good down to about I.Q.

TABLE 3—
*Fitting of the Negative Tail of the Frequency Curve of Stanford-Binet I.Q.'s*
(From Roberts, Norman and Griffiths, 1938)

| Percentile Point | I.Q. Range | Observed | Expected |
|---|---|---|---|
| | > 73.8 | 3,195 | 3,193 |
| 5 | 73.8–67.6 | 95 | 101 |
| 2 | 67.6–45.0 | 58 | 66 |
| 0.02 | < 45.0 | 13 | 0.7 |

45. Beyond that point, however, there is a very large excess of very low values.

If indeed the great bulk of high grade defectives owe their condition to the additive effect of many factors, some environmental, some genetic; and if, in fact, this part of the distribution of intelligence is only part of the distribution in the general population, then the regressions too should be similar. In the survey just mentioned the 269 children with the lowest 8 per cent of I.Q.'s (excluding the few idiots and imbeciles) had a mean I.Q. of 77.4. The mean I.Q. of their 367 sibs of school age was 88.1, giving a between sample regression of 0.53, a figure almost identical with that found in the population as a whole. Within the sample the regression was 0.49. In contrast, the I.Q. of the sibs of the idiots and imbeciles, who did not happen to include any very severely retarded children, was close to 100. Penrose (1939), starting with institutional defectives, obtained similar results. His defectives of I.Q. 50 plus gave a sib regression of 0.44. Taking his low grade group and omitting borderline subjects the mean I.Q. of sibs was 91.2. This last sample was much larger than the corresponding low grade sample in the Bath investigation, and it seems clear that the sibs of low grade subjects do on the average have I.Q.'s below 100. Part of this difference is no doubt due to bias in the selection of cases for institutional treatment; they tend to come from the poorer homes. But part may be real; it may perhaps reflect a tendency for those environ-

mental influences which are associated with low grade deficiency to produce also an average lowering of I.Q. in others.

Some brief mention may be made of an attempt to make a closer examination of the difficult borderline region, where, in terms of I.Q., there is much overlapping between the presumed low and high grade groups (Roberts, 1952). Two geographical areas were sampled as completely as possible, the group for study comprising children with I.Q.'s ranging from 35 to 60 on the Terman-Merrill scale. The number was 271 and the number of tested sibs was 562.

It was immediately apparent that the sample was very mixed up indeed; it could not be homogeneous. Nor, as had rather been anticipated, did the subjects of lower I.Q.'s within the selected range clearly belong to the presumed low grade group, and those of higher I.Q. equally clearly to the high grade group, with a mixed up part in the middle. There was too much overlapping for this.

An experiment was then tried. Looking at a two-way table showing the 271 subjects arranged vertically in order of I.Q., with the I.Q.'s of sibs alongside them, the families were divided quickly and arbitrarily into two groups. It should be mentioned that quite a number of families were ascertained through two, and occasionally more, defective children, and that the number of sibs was sometimes considerable. All such families had, of course, to be allotted to the one presumed group or the other. Table 4 shows in broad groups the I.Q.'s of subjects falling into the two

TABLE 4—*I.Q.'s of Subjects Falling into the Two Arbitrarily Selected Groups* (From Roberts, 1952)

| I.Q. | Imbecile Group | Feeble-minded Group |
|---|---|---|
| $< 53$ | 66 | 21 |
| 53–60 | 40 | 54 |
| $> 60$ | 16 | 74 |
| | 122 | 149 |

Note: I.Q.'s are adjusted to allow for differences in age at test. An incidental effect of the adjustment is to raise I.Q.'s by about 6 points on the average.

groups. In general there is a relation with I.Q., but clearly there is much overlapping.

The effect on the frequency distribution of I.Q.'s of sibs is shown in Figure 2. The whole sample of sibs gives a quite impossible frequency distribution, but the two groups separated in this way yield two quite reasonable curves. The sibs of the presumed feeble-minded group give a

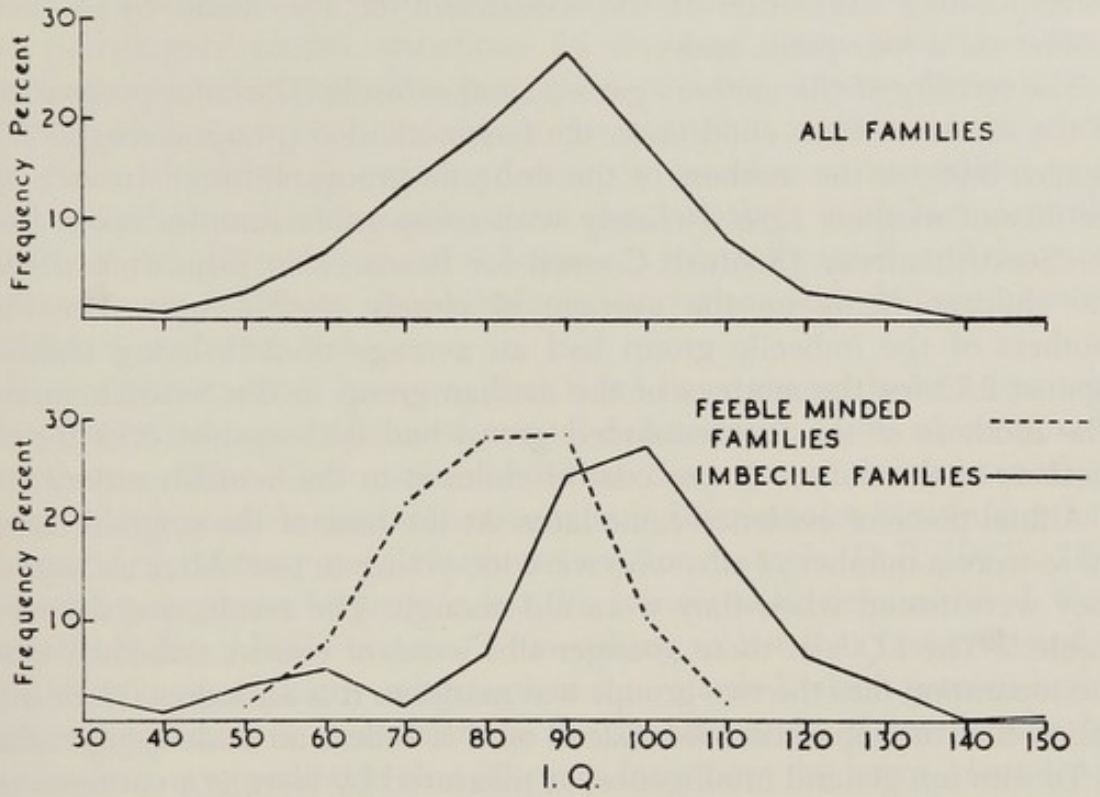


FIG. 2—Distribution of I.Q.'s of 562 sibs of mental defectives of I.Q. range 35-60 (from Roberts, 1952).

normal distribution centred on about I.Q. 80. The sibs of the presumed imbecile group give a curve centred on about I.Q. 100, but with a small although pronounced negative tail, which of course represents sibs of very low grade like the subjects.

Needless to say, the most difficult families to classify were those including only one tested sib. If these single sibs, 108 in number, are omitted the combined frequency distribution is little altered, but the lower curves are appreciably improved.

Turning to the regressions, the between sample regression shown by the feeble-minded group is 0.51, and the within sample regression 0.42. The within sample regression for the imbecile group is 0.21, not significantly different from zero.

These results seem to indicate that the arbitrary division into the two groups does correspond to an underlying biological reality. It would be very difficult to make an unreal arbitrary division which would satisfy all the criteria.

Some independent lines of evidence are available, however, on the reality of the difference between the groups. One is a relation to social class. Children of the imbecile group had fathers considerably higher in the social scale than had children of the feeble-minded group. There was a

corresponding difference in the assessment of the home by a social worker on a four point scale.

The fertility of the mothers gave a similar result. The total pregnancies of the mothers of the children in the feeble-minded group averaged 4.30, against 3.06 for the mothers of the imbecile group children. In fact, the fertility of mothers agreed closely with comparable samples taken from the Scottish survey (Scottish Council for Research in Education, 1949), the children being on the average of closely similar ages. Thus the mothers of the imbecile group had an average of 2.61 living children against 2.72 for the mothers of the median group in the Scottish survey. The mothers of the feeble-minded group had 3.31 against 3.72 for the mothers of the dullest 11 per cent of children in the Scottish survey.

A final piece of evidence came later. At the time of the original survey there were a number of sibs who were too young to test. After an interval they were tested when they were old enough. The results are shown in Table 5. The I.Q.'s of these younger sibs were, of course, unknown when the separation into the two groups was made, so it is an independent indication that the separation did indeed tend to reflect an underlying reality.

To sum up, general intelligence, as measured by tests, is a continuously distributed variable, giving a reasonably normal frequency distribution. The heritable part of the variation is considerable, amounting perhaps to something between a half and three-quarters. In terms of the metric employed inheritance is clearly multifactorial. At the extreme negative tail

TABLE 5—*Younger Sibs Subsequently Tested*

| I.Q. | Sibs of Imbecile Group | Sibs of Feeble-minded Group |
|---|---|---|
| 20-29 | 2 | |
| 30-39 | | |
| 40-49 | | |
| 50-59 | | |
| 60-69 | | 5 |
| 70-79 | | 6 |
| 80-89 | | 15 |
| 90-99 | 2 | 2 |
| 100-109 | 3 | 4 |
| 110-119 | 2 | |
| Total | 9 | 32 |
| Mean I.Q. | 104* | 83 |

* Omitting the two imbeciles.

there is an excess of very low values, representing deviations due to definite pathological causes, sometimes an abnormal gene, sometimes some accident of development. The proportion of these extreme deviants may be estimated as perhaps about four per thousand in children of school age. Some of the single causes produce on occasion no more than moderate retardation, but the indications are that their contribution to the sum total of high grade mental deficiency is relatively small, and the great bulk of such deficiency is multiple in causation, and that the genetic element is multifactorial.

It should be added, however, that there have always been a few who have queried the conception of mental deficiency in terms of continuous distribution. Thus Doll maintained that there is an innate something about a high grade mental defective which differentiates him from others of equal I.Q. who are not defectives (see, for example, Doll, 1947). That something may even be genetic, but this has yet to be demonstrated. Very recently Heber (1962) wrote: "I am willing to bet that factorial studies will identify many, many subgroups of the retarded which present highly different patterns of disabilities in the specific areas of intellectual behavior. It is my prediction that 20 years from now the terms Mental Age and I.Q. will be used only when speaking historically. The I.Q. and the M.A. and the total psychological test movement have served us well in our need for a gross classification instrument for more than 50 years. However, we are now beginning to develop psychological instruments which will provide a profile of levels of ability in the *many* specific intellectual areas."

Incidentally, it may be relevant to quote Masland (in Masland, Sarason and Gladwin, 1958). He does not believe that it is only the severe degrees of retardation which are associated with organic derangement or damage. He considers that the factor of brain damage operates throughout the whole range of intelligence and that minor degrees of damage are much more common than those that are severe and overt. This does not contradict the concept of continuous distribution, although it is difficult to fit in with the high degrees of heritability observed in the determination of intelligence level in general and in high grade mental deficiency in particular.

In all probability, however, most educational psychologists will continue to believe that the person of, say, I.Q. 60 to 70 who requires special treatment does so because of additional unfavorable social and family factors, and undesirable temperamental and emotional traits, and that an I.Q. does give a reasonable measure of intellectual retardation. It is this factor, as part of the general distribution of intelligence level, which shows so considerable a measure of heritability.

## Arterial Pressures and Hypertension

The analogy between I.Q. and mental deficiency on the one hand and arterial pressure and hypertension on the other is in many ways quite close. Benign essential hypertension must be accepted as a disease in that it is associated with increased levels of morbidity and mortality, which display, moreover, characteristic patterns. Yet essential hypertension is of all human diseases the one which is most purely defined by a measurement, namely, level of arterial pressure. Until comparatively recently the difference between hypertension on the one hand and normality on the other was always thought of in qualitative terms. This led to many attempts to fix a dividing line in terms of arterial pressure. It is an attempt fraught with difficulty. As Pickering (1961) has pointed out, every round value (and some intermediate ones) from 120 systolic and 80 diastolic to 180 systolic and 110 diastolic has been suggested at some time or other.

It was also clear that the degree of heritability was at least moderately large, and perhaps very large. As long ago as 1923 Weitz suggested a dominant gene. He measured the pressures in 93 sibs of 42 patients with essential hypertension. Confining attention to 47 sibs older than the subject he found the ratio of those affected to those unaffected was 20:27 if 150 mg.Hg systolic was taken as the dividing line, and 18:29 if 160 mm. Hg was selected. Eleven sibs born before the subject had died of heart disease or strokes, and if they were accepted as probable instances of essential hypertension the theoretical ratio of 1:1 was closely realized. A number of other studies were carried out on similar lines, and in general led to the same conclusion. The most comprehensive study was that of Søbye (1948). He adopted a cut-off point of 160/100 and found that a dominant gene hypothesis fitted.

An alternative explanation was put forward by Hamilton, Pickering, Roberts and Sowry (1954a, 1954b, 1954c). They had made observations on three samples:

1. A sample intended to represent the general population and comprising 2,031 subjects.
2. A sample of 109 subjects suffering from essential hypertension. All except two had diastolic pressures of 100 mm. Hg or more. 387 first-degree relatives were measured.
3. A control sample of 102 subjects without essential hypertension. In these subjects the diastolic pressure had not been known to have exceeded 85 mm. Hg. 371 first degree relatives were measured.

The first and most important finding which led to the formulation of a quantitative hypothesis was that the pressures of the relatives of the

hypertensives exceeded, on the average, those of the control relatives, and those of the population sample, at all ages, down to the age group 10-19 years, the youngest represented.

The qualitative and the quantitative hypotheses necessarily lead to different treatments of age differences, for, on the average, although only on the average, the level of arterial pressure rises with age. On the qualitative hypothesis age may be regarded as in a sense irrelevant (although some would concentrate on the limits of, say, 45-59 years [Platt, 1959]), but clearly the proportion of those formally judged to be suffering from hypertension increases with age. The appropriate adjustment, as made by Søbye for example, is to compare rates of manifestation by age and sex amongst relatives with those observed in the general population. The quantitative hypothesis demands a different approach. It is necessary to compare subjects and relatives of different ages and of both sexes. A reasonable measure is the deviation from the norm as established from the general population, with a further adjustment to allow for the increase of variance of pressure with age. Hamilton et al. (1954b) devised age and sex adjusted scores which were simply the deviations in mm. Hg, plus or minus, from the norms established on the basis of the population sample, multiplied by the ratio of the standard deviation at age 60 to the standard deviation at the observed age. This age was chosen because at about 60 years the variance in the two sexes is nearly equal, both for systolic and for diastolic pressures; hence adjustment is also made for difference in sex. The scores are, in fact, similar to the standard scores of the educational psychologist, being proportional to units of the standard deviation.

Using the scores it was found that the degree of resemblance of relatives to subjects was indeed not significantly different at different ages, being the same for fathers, mothers, brothers, sisters, sons and daughters. In fact, the resemblance between relatives could be represented by a single regression coefficient of a little more than 0.2, and this applied over all ages and at all levels of pressure, both in the control sample and in the hypertensive sample, except that there was some tendency for the regression to diminish with those hypertensive subjects having the highest scores. A re-examination of Søbye's figures, which are given in detail in his monograph, gave closely similar results.

That the scores do tend to reflect genetic reality is shown by the figures of Table 6. The actual pressures of the hypertensive subjects were not greatly different at different ages, but naturally the age and sex adjusted scores were very different, young subjects giving very high scores and old subjects much lower ones. But this is faithfully reflected in the scores of the relatives, and the regressions are strikingly uniform. Young subjects

TABLE 6—*Arterial Pressures and Age Adjusted Scores of Propositi by Age, with Scores of Relatives, and Regressions*
(From Hamilton, Pickering, Roberts and Sowry, 1954c)

| Age of Propositus | No. of Propositi | Actual Mean BP Propositi | Mean AAS Propositi | No. of Relatives | Mean AAS Relatives | Regressions* | |
|---|---|---|---|---|---|---|---|
| | | | | | | Systolic | Diastolic |
| 20-34 | 20 | 190/119 | 136/70 | 61 | 28.7/14.3 | .21 | .20 |
| 35-49 | 49 | 214/129 | 111/58 | 171 | 25.0/13.7 | .23 | .24 |
| 50-64 | 34 | 225/129 | 87/45 | 126 | 20.0/10.6 | .23 | .24 |
| 65-79 | 7 | 200/118 | 26/24 | 29 | 10.3/ 6.2 | .40 | .26 |

* Between sample regressions obtained by dividing mean age-adjusted score of relatives by mean age-adjusted score (AAS) of propositi (unweighted).

with very high pressures differ widely from the norm, older subjects less so. But their relatives show corresponding deviations. The average pressures of relatives of these young hypertensive subjects is much higher than that of the relatives of the older hypertensives. As would be expected on the quantitative hypothesis there are degrees of hypertension and except perhaps at the highest levels this is mirrored in the pressures of relatives.

A word might be said here about the form of the frequency distributions of arterial pressure. On a linear scale they are markedly non-normal, showing pronounced positive skewness. Gaddum (1948), using the data of Alvarez on University freshmen, showed that on a logarithmic scale the distribution was quite closely Gaussian. He has pointed out that a log normal distribution is much more often characteristic of biological measurements than is normality on a linear scale (Gaddum, 1945). The data of Hamilton et al. show the same thing. The frequency distributions on a logarithmic scale are very nearly Gaussian. It is difficult to secure large numbers having a narrow range of age, but using the scores and adding to expectation at age 60, it was found that the distributions were closely log normal in the population sample, and also in the relatives of hypertensives, whose whole distribution curve is shifted to the right. It should be pointed out, however, that the use of the scores in this way has a smoothing effect and that, in particular, bimodality might be concealed, a subject mentioned later. The enormous numbers of Bøe, Hummerfelt and Wedervang (1957) in the Bergen survey, permit a closer analysis in

terms of actual pressures. An examination of their data shows that the frequency curves are symmetrical on the log scale up to about age 35; thereafter there is moderate positive skewness which disappears again in the oldest age groups.

The control sample of Hamilton et al. was inadequate in numbers and not entirely satisfactory in its method of selection. Hence some conclusions, particularly in regard to resemblances between relatives at lower levels of pressure, were necessarily somewhat tentative. The gap has been most adequately filled by magnificent surveys on the general population carried out by Miall and Oldham (1955, 1957, 1958, 1963). Truly random samples were selected from two ideal "captive" populations and the attempt made to measure all first-degree relatives living within 25 miles of the areas in question. The sampling was very complete, more than 95 per cent of subjects and relatives being measured. In a follow up of 4 years later, more than 98 per cent of the original sample was measured. The total number of subjects was more than 600, and of first degree relatives more than 2,200. Age and sex adjusted scores were used, as by Hamilton et al. (1954b). In the paper of 1963 Miall and Oldham showed how the linearity of the regressions improved (1) as the original sample was doubled in size, (2) on taking the mean of the two measurements separated by a 4-year interval. This last procedure, of course, removes some of the variation due to diurnal fluctuations. The final result is shown in Figures 3 and 4. There is no evidence of any significant departure from linearity from the lowest pressures to the highest. The estimates of the regressions, based on a single measurement, are very similar to those deduced from the smaller samples of Hamilton et al. They are $0.224 \pm 0.022$ for systolic pressures, and $0.178 \pm 0.024$ for diastolic pressures.

The remeasurement after the lapse of 4 years provided material for some interesting calculations. Taking the mean of the two measurements,

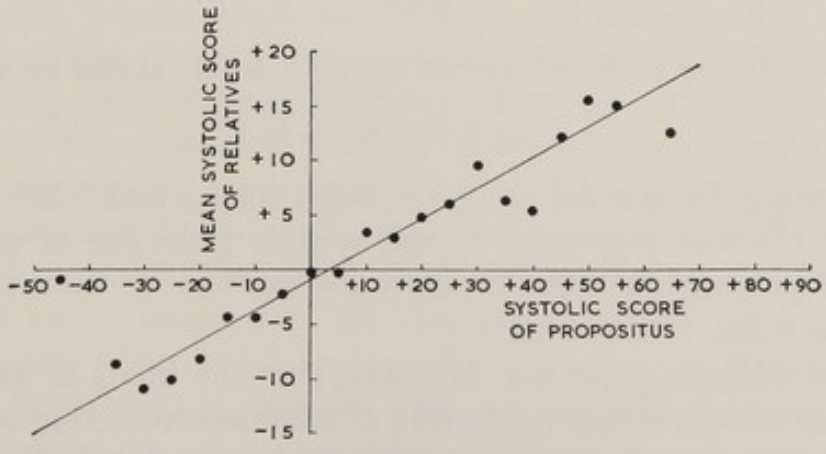


FIG. 3—Relationship between the systolic blood pressure scores of propositi and the mean systolic scores of relatives (from Miall and Oldham, 1963).

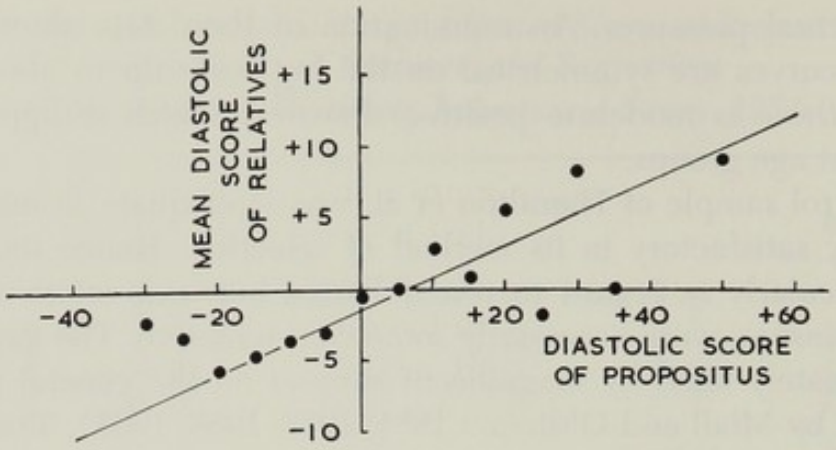


FIG. 4—Same as Figure 3, but for diastolic blood pressure scores (from Miall and Oldham, 1963).

the systolic regression became 0.287 and the diastolic 0.224. Miall and Oldham point out that taking the mean of the two measurements must have approximately halved the variance due to the random or observational element, which can be thought of as additional to the variance between subjects which reflects real differences in pressure. They show that if c is the covariance due to the resemblance of relatives, $\sigma^2$ the true variance, and $s^2$ the additional error variance (both $\sigma^2$ and $s^2$ being similar in the two surveys), the regression of relatives on propositi, $b_1$, observed in the first survey will be

$$c/(\sigma^2 + s^2)$$

while the regression, $b_2$, observed in the two surveys pooled will be

$$c/(\sigma^2 + \tfrac{1}{2}s^2)$$

It is therefore possible to estimate the value b, equal to

$$c/\sigma^2$$

that would be found if there were no error variance. It can be shown that

$$c/\sigma^2 = b_1 b_2/(2b_1 - b_2)$$

and substituting, for systolic pressures, 0.224 for $b_1$, and 0.287 for $b_2$, the estimate of the true regression of the systolic pressures of first degree relatives on propositi is 0.399. Similarly the estimate of the true diastolic regression is 0.302.

Miall and Oldham point out, however, that the limits of error of the estimates are wide and that similarities of environment acting within families may be inflating the covariance. It may be unwise, they state, to remove nongenetic variance from the denominator of the ratio giving the

regression while leaving the numerator unchanged. The calculation is in fact an interesting speculation, and they consider that the uncorrected regressions, based on pooling the two measurements, may be the more reliable figures to use.

Miall and Oldham use their figures to estimate heritability. The square of the correlation of 0.287 (systolic) between first degree relatives, 0.082, estimates the proportion of variance accounted for by one parent, and an equal amount can be attributed to the other. If hereditary determination were complete (on the simplified model) 50 per cent of the variance can be accounted for in this way, hence hereditary determination corresponds to 33 per cent, leaving 67 per cent as presumably environmentally determined. The 95 per cent fiducial limits of this last figure are 56 and 77 per cent. The corresponding estimate for the environmental element in diastolic pressure is 80 per cent, with fiducial limits 71 and 87 per cent. If the estimated error-free regressions of 0.399 systolic and 0.302 diastolic are used the proportion of the variance due to nonfamilial environmental factors would be, respectively, 36 per cent and 64 per cent.

The parent and child and the sib regression coefficients are as follows:

| | Systolic | Diastolic |
|---|---|---|
| Parent and child | 0.237 | 0.183 |
| Sibs | 0.333 | 0.265 |

The ratio of dominance, H to D and of E (environmental factors) to D can be estimated from these figures. They come out as follows:

| | H/D | E/D |
|---|---|---|
| Systolic | 1.620 | 0.150 |
| Diastolic | 1.792 | 0.418 |

This seems an impossible result, as the contribution of dominance is so large. Probably wisely, Miall and Oldham did not give this calculation in their paper. The probable explanation of this curious result is the very large sampling errors of these estimates. Moreover, as they are ratios, the fiducial limits are asymmetrical, large positive values being as likely to occur as small negative ones. In addition to this, a greater relevant environmental similarity between sibs than between parent and child would appear as dominance. This result illustrates the rather limited practical use of these ratios, unless data are very ample indeed.

An interesting point which emerges from this survey is the clear indication that heritability is stronger with systolic than diastolic pressures. This rather surprising result cannot be attributed to any greater difficulty of taking accurate diastolic readings, for this factor would have come

out in the calculations of Miall and Oldham as given above. It may be, perhaps, an interesting starting point for future researches.

In regard to the metric, level of arterial pressure, as observed in the general population, the conclusion seems reasonably clear. It is continuously distributed and shows a moderate degree of heritability, which is expressed, in terms of a reading on a single occasion, by a correlation of about 0.2 between first degree relatives. Multifactorial determination of the hereditary component seems to be the obvious hypothesis. But how far can this be extended to cover essential hypertension, as originally suggested by Hamilton et al.? There are those who maintain strongly that hypertension is a qualitatively distinct entity, and also (although this is not a necessary corollary) that it is very largely hereditary and determined by a single dominant gene.

First of all, it must be recognized that hypertension is a very common disease. Thus Søbye (1948) estimates its frequency as 30-40 per cent in those persons who live long enough. Platt (1959) gives 19-30 per cent.

Clearly, if the criteria are such that so large a proportion of the population must be judged abnormal, then complete population studies like those of Miall and Oldham must include a considerable proportion of persons who have essential hypertension. It is very difficult to see how this can be fitted in with a qualitative hypothesis.

The presumed dominant gene must be correspondingly common, and so great is the estimate of heritability if a usual cutoff point, say 160/100, is chosen that there is practically no room for lack of penetrance. Diurnal variation is very great, and whatever cut-off point is selected, there must be a large measure of misclassification. It is difficult to see how this can be fitted in without assuming a heritability which is impossibly high. Nor can it be doubted that environmental factors must play some part, and there hardly seems room for them. It must be a very remarkable gene, probably unique in genetics. And how could so harmful a gene have a frequency in the population of 30 per cent or so? No counterbalancing advantage has ever been suggested. It has been said that a gene which produces its effect only after reproductive life is largely over is not subject to natural selection. Theoretically this may be true, but experience both with man and in experimental animals does not suggest that the common diseases of postreproductive life display Mendelian ratios.

Those who favor a quantitative view do not contend that all benign essential hypertension necessarily displays, in regard to its heritable component, a multifactorial basis. There may be single genes, or particular single environmental influences, which produce hypertension, but if so it seems inescapable that their contribution to the total bulk is relatively small, as with high grade mental deficiency.

There has been considerable discussion as to whether the frequency distributions of arterial pressures provide evidence of bimodality. It is, of course, a difficult question to investigate unless the bimodality is pronounced. In samples from the general population there is certainly no clear evidence of bimodality, provided the samples are large enough, as of course they must be. The remaining positive skewness, even on a logarithmic scale, between, say, ages 35 and 65 does, however, leave some room for doubt. The argument is related particularly to the frequency distributions of first degree relatives of hypertensives. Platt (1959) pointed out that the frequency distributions given by Hamilton et al. (1954c) and Søbye (1948) did show pronounced bimodality, with the dip at about the level of 150 mm. Hg systolic. This was countered (Oldham, Pickering, Roberts and Sowry, 1960) by the suggestion that in some part this might be due to digit preference, for with most observers reading to the nearest 5 mm., 0's appear more often than 5's. Much more important, however, is the possibility of conscious or unconscious bias, that is, the anxiety of the observer to assign a subject definitely to the normal or to the hypertensive range. This is particularly true of the data of Søbye, for he made repeated measurements, and finally selected one which he considered represented the best reading for the subject. It is also possible that some degree of bimodality might be due to a tendency for pressures to rise unduly fast over the range of, say, 140-160 mm. Hg systolic, and correspondingly for diastolic pressures. It should be mentioned, however, that the remeasurement data for Miall and Oldham (quoted by Oldham et al., 1960) do not support this suggestion. It is difficult to summarize a complicated argument fairly, but it would seem that bimodality is not a pronounced feature and that this line of evidence for the existence of two populations is not convincing. Furthermore, the remeasurement data just mentioned give no indication of two populations growing apart during a critical range of age.

It is always open to the opponent of the quantitative hypothesis to claim that the measurement does not really represent the disease. The similar contention with I.Q.'s and mental deficiency has already been mentioned. At present, however, it does not seem that any authority is prepared to define the disease in terms independent of the level of pressure.

A final word might be said about malignant and secondary hypertension. Hamilton, Pickering, Roberts and Sowry (1963) have presented data on the pressures of first degree relatives of such subjects, obtained in just the same way as with essential hypertension. The relatives of propositi with malignant hypertension show a frequency distribution of pressures which is closely similar to that of relatives of propositi with benign

essential hypertension. This is not very surprising, perhaps. It fits in with the view that the malignant phase may supervene whenever the pressure rises high enough and fast enough, whatever the cause of the rise. A full discussion of this question is given by Pickering (1955, 1961). It is also to be noted that the very high pressures of subjects with malignant hypertension is not reflected by a corresponding very high average pressure in the relatives. It looks as though the passing of the disease from the benign to the malignant phase is not particularly mediated by genetic influences.

The surprising result obtained by Hamilton et al. (1963) is that the distribution of pressures of relatives of subjects suffering from pyelonephritis (and also from hypertension, for that was the way the sample was chosen), is also closely similar to the distribution of relatives of subjects with benign essential hypertension. Some alternative hypotheses were put forward to account for this finding, one possibility being that those who suffer from pyelonephritis are those with the constitutional tendency to experience hypertension. But further work is needed before the different hypotheses can be adequately tested.

## Other Conditions Giving Some Indications of Quantitative Variation

Some brief reference may now be made to conditions in which the quantitative element in the observed disease is less obvious, and yet there are some indications of it. Quite possibly diabetes mellitus may one day fall into this category. Despite much genetic work over many years the role of inheritance in this disease remains obscure, although clearly it is important. Almost all observers tend to invoke a gene, or alternative genes, with somewhat low penetrance. A favored hypothesis is a recessive gene, of low penetrance, perhaps about 10 per cent. The reason for this is the fact that in diabetes, affected relatives are so often found on both sides of the family. There are complications, however. One is the question of whether in general, diabetes is one condition or not. Some would distinguish between diabetes of the young and diabetes of the middle-aged and elderly. The consensus, however, seems to favor a unitary hypothesis, for it is often found that diabetes of the elderly is found in relatives of those suffering from childhood diabetes, and vice versa. As so often happens, increasing manifestation with age raises technical problems which are not easily solved.

A recessive gene of low penetrance appears to be something of a rarity, for the expression of recessive genes tends to be much more con-

stant than the expression of dominant genes in the heterozygote. Some studies on diabetes point to a quantitative element. Relatives are noted who show glycosuria without any overt disease. Moreover, despite attempts to assign blood sugar curves into the two categories, normal and abnormal, the curves of relatives of diabetics do not simply show an excess of frankly, abnormal values, but also a general shift in the positive direction. Thus, a maximum of value, say, 160 or 170 is commoner than in the general population (Keen, 1963).

With diabetes anything measurable tends to show extreme deviations in those with established disease, but it does seem possible that the genetic element in causation may ultimately turn out to be multifactorial. But, as with other diseases, this does not mean that diabetes might not on occasion have a different genetic basis, and perhaps a relatively simple one involving single major genes.

In congenital dislocation of the hip there is a measure of genetic determination, but in a careful twin study it was shown that less than half the monozygotic pairs were concordant (Idleberger, 1951). The incidence among relatives is quite low. A number of environmental factors are known to affect the incidence of the condition. These are discussed by Record and Edwards (1958). For many years the favored explanation of the genetic basis of congenital dislocation of the hip was a dominant gene of reduced penetrance, but Record and Edwards pointed out that the genetic element is essentially related to factors affecting the stability of the hip joint, and that from the quantitative nature of the variation in this respect genetic determination is likely to be multifactorial. Wilkinson and Carter (1960) and Carter and Wilkinson (1963) consider that there are three important factors in the causation of congenital dislocation of the hip. These are (1) width of the acetabular angle, (2) generalized joint laxity, (3) breech presentation. They present evidence which points to generalized joint laxity being simply determined genetically, perhaps by a dominant gene. Width of acetabular angle, however, is a graded character. Increasing shallowness of the joint socket brings increasing likelihood of dislocation and they suggest that this important element in genetic predisposition is multifactorial in its determination.

It may be, perhaps, that some mental diseases will ultimately fall into the same category. A single gene basis (or several alternative genes) has always seemed improbable to some as the genetic element in schizophrenia. The question of frank disease and of schizoid personality is complicated, however, and resolution of these difficulties must be left to the psychiatrists.

Thus it may be that with a number of diseases which are essentially of an all or none character there yet may be indications of lesser degrees of

abnormality or of variation in something closely related, with the suggestion that it is this quantitative element which is involved in hereditary predisposition. As these diseases become better understood, the quantitative element may well become increasingly evident, making it likely that determination, to the extent to which it is genetic, is multifactorial.

## Common Diseases

Many common diseases show evidence of a measure of genetic determination. There is an excess of affected persons among relatives which cannot easily be accounted for by environmental similarities. In the past single genes of low penetrance have often been invoked, but this seems less common today. This is not because a single gene hypothesis will not fit, but because a multifactorial basis fits equally well, and seems inherently more probable.

With genetic differences in susceptibility and resistance to infective diseases, studies on experimental animals nearly always seem to point to a multifactorial basis. It may be recalled, moreover, that sometimes what is really important is not whether the body is invaded by a microorganism, but the differences in the reaction of the host after infection has taken place. This is very true of tuberculosis, for example.

With neoplastic diseases there are a few in which determination, or at least the determination of a precancerous condition, is simply genetic. There are also individual families which show so high a concentration of some otherwise unremarkable cancer that coincidence seems very improbable, and a strong genetic element must be presumed. Such families are very rare, however. The great bulk of neoplastic disease seems either to show a relatively small increase in the incidence of the same type of cancer in relatives, or sometimes there is no evidence of any increase at all. This contrasts sharply with what is found in particular strains of experimental animals, some being highly susceptible to one kind of cancer, others to another. This may well be a likely consequence of a measure of multifactorial determination. In a more or less randomly breeding species such as man, a large part of the genetic variation is concealed. Mather (1960) points out that the proportion of the variation which is concealed is (k-l)/k, where k is the number of loci involved. Thus, with 10 gene pairs, 90 per cent of the variation is concealed; with 100 pairs, no less than 99 per cent. In the development of highly inbred experimental strains this variation is unscrambled, and so it may be that concentrations of genes making for a particular high susceptibility become stabilized. This is something that does not happen in a randomly breeding population.

If this conception of a measure of multifactorial determination in many diseases is correct it provides an example of meristic, or quasi-continuous, variation. Underlying degrees of resistance and susceptibility are continuously distributed, but the appearance of the disease is all or none. Whether or not a given individual suffers from the disease depends upon his level of constitutional susceptibility, together with the environmental factors which are involved in causation. As indicated in the previous section, it may be that the nature of the underlying constitutional susceptibility can sometimes be elucidated, and might then be seen to be continuously distributed. But often this will not be so; all we see is the presence or absence of the disease.

As single gene hypotheses for the hereditary element in the causation of common diseases seem to be invoked with increasing infrequency, it may suffice to give a single example in which the effect of two known major gene loci can be estimated. It has long been known that in duodenal ulceration there are more similarly affected close relatives than is found in the general population. A careful study by Doll and Buch (1950) provides admirable figures. They found that 45 out of 562 brothers of men with duodenal ulcer were similarly affected. The expected number, based on a comparable control population and matched by age was 16.67. Thus, the incidence among brothers was 8.0 per cent as against an expectation of 3.0 per cent, or 2.7 times as great. Doll and Buch concluded that similarity of environment was very unlikely to account for this difference, or indeed for any substantial part of it; it must be assumed to be largely genetic.

It is now known that duodenal ulceration shows an association with the ABO blood groups and with secretor status (for a general review, see Clarke, 1961). As the result of the work of a number of investigators the relative incidence of duodenal ulceration in the different phenotypes can be quite closely estimated. The figures of Table 7 are taken (without

TABLE 7—*Relative Incidence of Duodenal Ulceration in Subjects of the Four Phenotypes*

| Phenotype | Relative Incidence |
|---|---|
| Secretor, not-O (SN) | 1 |
| Secretor, O (SO) | 1.4 |
| Nonsecretor, not-O (sN) | 1.8 |
| Nonsecretor, O (sO) | 2.7 |

weighting) from the data given by Doll, Drane and Newell (1961). Doll et al. show that the effects of blood group and secretion are, in fact, multi-

plicative and not just additive. In susceptibility to duodenal ulceration there is no difference between persons of groups A, B and AB. Hence, it is sufficient to think simply of O and not-O, not-O, of course, being dominant to O. Secretion is dominant to nonsecretion. The loci do not show genetic linkage.

TABLE 8

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| Phenotype | Population Frequency | X Factors of Table 7 | Expected Frequencies of Those with DU | Expected Frequencies in Brothers | X Factors of Table 7 |
| SN | .3825 | .3825 | .262 | .326 | .326 |
| SO | .3675 | .5145 | .353 | .368 | .515 |
| sN | .1275 | .2295 | .158 | .143 | .257 |
| sO | .1225 | .3308 | .227 | .163 | .441 |
| | | 1.457 | | | 1.539 |

The frequencies of the genes in the English population are quite close to: 0(0), 0.7; not-O(N), 0.3; secretors(S), 0.5; nonsecretor(s), 0.5. These are about the expected frequencies in Northern England. The frequences of the four phenotypes can now be calculated; they are shown in the second column of Table 8. Multiplication by the factors for relative incidence shown in Table 8 gives column 3, which gives the relative frequencies of the four phenotypes in persons suffering from duodenal ulcer. The total is reduced to one in column 4, which may be compared with the population frequencies of column 2. The figures of column 4 are, in fact, very close to the observed frequencies in patients with duodenal ulcer. Given these frequencies, it can be calculated what the frequencies of phenotypes will be among brothers on the assumption of random mating. The result is shown in column 5. Again, multiplying by the factors of Table 7, column 6 is obtained and its total may be compared with the total of column 3. The ratio of the two totals is 1.056:1.

Thus the incidence of duodenal ulceration among the brothers of affected men is increased as the result of genetic similarity at the two loci by only 5.6 per cent as against the incidence in the general population. These two major gene loci are contributing as a secondary effect to a multifactorial system which must include many more genes; some of these may be major genes with side effects, like the ABO and secretor genes; but most, in all probability, are the usual polygenes of small effect.

## Congenital and Allied Malformations

Many congenital and allied malformations, apart from that small proportion which is simply genetic, show evidence of partial genetic determination. There is often a higher concordance rate between monozygotic as against dizygotic twins. There is frequently an increased incidence among relatives, although the increase is often relatively small. As always, the mere analysis of these frequencies does not provide tests for deciding whether the genetic element is a major gene of reduced penetrance, or is to be ascribed to multifactorial inheritance. Yet, with definite, all or none malformations, often with little or no indication of minor unimportant *formes frustes*, the major gene of reduced penetrance seemed on a priori grounds to be the more likely hypothesis. Indeed, when genetic hypotheses have been formulated they have practically always been of this type. Yet even with congenital abnormalities, evidence is now emerging that the genetic basis may sometimes after all be multifactorial.

The chief contribution has been made by Carter (1961a, 1961b, 1963) in studies on congenital pyloric stenosis. This is a relatively common condition, having an estimated frequency in England of about three per 1,000 live births. The sex ratio is very unequal, being about five boys for every girl. Thus the estimated population frequencies are about one per 200 male live births and one per 1,000 live female births. Prior to the introduction into Great Britain of Rammstedt's operation at about the end of the first World War mortality was very high, so it was many years before

Table 9—*Congenital Pyloric Stenosis. Incidence in Sibs and Offspring of Affected Persons* (Carter's data, 1963)

| | Affected | Total | % Affected |
|---|---|---|---|
| | | Relatives of Affected Men | |
| Brothers | 5 | 184 | 2.7 |
| Sisters | 5 | 195 | 2.6 |
| Sons | 15 | 250 | 6.0 |
| Daughters | 6 | 234 | 2.6 |
| | | Relatives of Affected Women | |
| Brothers | 7 | 49 | 14.3 |
| Sisters | 2 | 48 | 4.2 |
| Sons | 11 | 50 | 22.0 |
| Daughters | 6 | 51 | 11.8 |

A more detailed comparison is shown in Table 10.

data became available on offspring of affected persons. Knowledge was confined to sibs and it was reasonable to put forward the tentative hypothesis of a recessive gene of quite high penetrance in boys and much lower penetrance in girls (Cockayne and Penrose, 1943). Later, when children of affected persons began to appear, it was found that the incidence in children was as high as in sibs (Carter and Powell, 1954).

Carter's systematic studies (1961a, 1961b, 1963) started with all patients operated on at The Hospital for Sick Children, Great Ormond Street, between 1920 and 1939. A very high proportion was successfully traced, and all those having one or more children were visited. The results, in terms of affected relatives, are shown in Table 9.

TABLE 10—*Congenital Pyloric Stenosis. Comparison of Proportions Affected in Relatives of Affected Males and Affected Females*
(Carter's data, 1963)

| | Percentages | | | | |
|---|---|---|---|---|---|
| | Relatives of Affected Men | Relatives of Affected Women | Differences | S.E. | Diff./S.E. |
| Brothers | 2.7 | 14.3 | 11.6 | 5.1 | 2.3 |
| Sisters | 2.6 | 4.2 | 1.6 | 8.1 | 0.2 |
| Sons | 6.0 | 22.0 | 16.0 | 6.0 | 2.7 |
| Daughters | 2.6 | 11.8 | 9.2 | 4.6 | 2.0 |

Three of the four comparisons give a difference which is individually significant at the 5 per cent level. If the data are combined into a single comparison the difference is highly significant, p being less than one in 1,000.

One point should be made clear. The incidence in offspring is higher than the incidence in sibs. As shown in Table 11, however, the apparent differences are not significant. Nor does the difference reach significance at the 5 per cent level if the data are combined to give a single comparison. A higher incidence in offspring than in sibs would be very difficult to explain, but it would seem that this is unnecessary, and that the probable reason for it is chance variation.

The findings in congenital pyloric stenosis are impossible to interpret in terms of a single gene of lowered penetrance; and, incidentally, sex linkage can be ruled out. Girls who are affected are abnormal despite the protection afforded by their sex, and this is faithfully mirrored in the higher incidence among their sibs and children as compared with the incidence among the sibs and children of affected boys. Thus degrees of genetic susceptibility must be quantitatively distributed.

TABLE 11—*Congenital Pyloric Stenosis. Comparison of Proportions of Sibs and of Children Affected*

| | Percentages | | | | |
|---|---|---|---|---|---|
| | Affected Sibs | Affected Children | Difference | S.E. | Diff./S.E. |
| Sons and brothers of affected men | 2.7 | 6.0 | 3.3 | 1.9 | 1.7 |
| Sisters and daughters of affected men | 2.6 | 2.6 | 0 | — | — |
| Brothers and sons of affected women | 14.3 | 22.0 | 7.7 | 7.7 | 1.0 |
| Sisters and daughters of affected women | 4.2 | 11.8 | 7.6 | 5.4 | 1.4 |

Carter's work does indeed seem to be a major advance in the very difficult task of distinguishing between single factor inheritance and multifactorial inheritance from the limited data which are all that can be assembled in practice when dealing with human material. The opportunity was provided by the wide difference in sex incidence and some further suggestive findings will be mentioned immediately. It should be possible in future to use other nongenetic differences (if sex differences can be called nongenetic) when these have been established. If, for example, a high incidence is found in one particular group and a low incidence in another, then if multifactorial inheritance is involved affected persons belonging to the favored group may show higher incidences in relatives than those coming from the less favored group.

There are some indications that harelip (with or without cleft palate) may show the same pattern of genetic behavior as pyloric stenosis. Fogh-Andersen's (1943) comprehensive survey gives excellent risk figures for relatives, and these are in good agreement with the findings in other studies. One feature which emerges from his work is that in those families in which there are two or more affected relatives, other than sibs, the proportion in which the affected relatives occur on both sides, that of father and of mother, is too high to fit the hypothesis of a dominant gene of partial penetrance. Fogh-Andersen noted this, although the comparison he shows is somewhat obscured by adding all those families in which there was only one affected relative, who must, of course, be on one side or the other. Removing these the comparison is shown in Table 12. To his figures can be added those of Roberts, Carter and Buck (1963). These represent a part of the material which has so far been analyzed. Clearly,

TABLE 12—*Harelip and Cleft Palate.*
*Families in which there are Two or More Affected Relatives Other Than Sibs*

| | Affected on One Side of Family Only | Affected on Both Sides of Family | Total |
|---|---|---|---|
| Fogh-Andersen (1943) | 29 | 6 | 35 |
| Roberts, Carter and Buck (1963) | 12 | 4 | 16 |
| Total | 41 | 10 | 51 |

the proportion of families with affected relatives coming from both sides is much too high for a dominant gene. In fact, a recessive gene of low penetrance has been suggested on several occasions.

Fogh-Andersen's data were incomplete in one respect. He had very few children of index cases and so his estimate of incidence in offspring, derived from the children of affected persons other than propositi, was very tentative. One of the objects of the survey of Roberts, Carter and Buck (1963) was to fill in this gap. The procedure was the same as with pyloric stenosis, namely, to start with children treated at The Hospital for Sick Children between 1920 and 1939. As many as possible were traced and all those who had children visited. In fact, the estimate for children turned out to be very similar to Fogh-Andersen's tentative figure. The incidence in offspring is very similar to that in sibs. This contradicts the hypothesis of a recessive gene.

That one line of evidence should point to a dominant gene, and another to a recessive gene, alone seems to make any major gene hypothesis unlikely. Another finding also seems to fit multifactorial inheritance more easily than it does any alternative hypothesis. This is that when an affected person has had an affected child, the risk to subsequent offspring is much increased, rising to 8 or 10 per cent. On a multifactorial hypothesis this could be explained by the birth of an affected child pointing, on the average, to a higher degree of susceptibility; and also to a high degree of susceptibility coming in from both sides.

There is one further piece of evidence. With harelip and cleft palate the sex incidence is unequal, being about two boys to one girl. The difference is much less than with pyloric stenosis, and as the incidence among relatives is also lower, much larger numbers would be required to establish the same point. Nevertheless, the findings of Roberts, Carter and Buck do give some suggestive indication of the same phenomenon. Taking the sons of affected men and women, respectively, the results are as follows:

| | Affected | Total | % Affected |
|---|---|---|---|
| Sons of affected men | 6 | 183 | 3.3 |
| Sons of affected women | 10 | 114 | 8.8 |

The difference is not significant, $\chi^2$ being 3.15, but at least it is a deviation in the same direction as with congenital pyloric stenosis. Other relatives, numbers being smaller, do not give any clear result. So there is the suggestion that ultimately harelip and cleft palate too, may yield evidence for a multifactorial basis as against a single gene, at least in some part of the material, for it is probably not homogeneous.

Another suggestive finding may be quoted. In talipes equinovarus there is again an excess of males, the ratio being about two males to one female. Wynne-Davies (1963) has obtained the results shown in Table 13.

TABLE 13—*Talipes Equinovarus. Incidence in Relatives of Affected Persons* (From Wynne-Davies, 1963)

| | Relatives of Affected Men | | | Relatives of Affected Women | | |
|---|---|---|---|---|---|---|
| | Affected | Total | % Affected | Affected | Total | % Affected |
| Brothers | 4 | 115 | 3.5 | 2 | 33 | 6.1 |
| Sisters | 0 | 90 | 0 | 2 | 34 | 5.9 |
| Fathers | 1 | 97 | 1.0 | 3 | 47 | 6.4 |
| Mothers | 0 | 97 | 0 | 0 | 47 | 0 |
| Total | 5 | 399 | 1.3 | 7 | 161 | 4.3 |

The differences are in the direction suggested by the sex ratio on the assumption of multifactorial inheritance, the inherently less susceptible affected girls having more affected relatives. If a single combined comparison is made of relatives of males and of females, respectively, the difference just attains significance at the 5 per cent level.

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Chapter 7

# The Quantitative Assessment of Hereditary Damage Induced by Radiation

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There is little direct information about the hereditary damage produced in man by ionizing radiation. On the other hand, an overwhelming mass of data on the genetic effects of radiation on experimental organisms compensates to some degree for this shortage. These data have been ample to establish a set of basic principles concerning the qualitative nature of radiation-induced damage and there is seldom any hesitancy in applying these principles to man.

Perversely, the abundance of data from other organisms has failed to provide information that can be applied quantitively to man with any degree of assurance, and estimates of the magnitude of the genetic consequences of irradiation of human populations remain disturbingly uncertain. Recent investigations have been most successful in emphasizing the inadequacies of present methods of assessment. Indeed, it even seems sometimes that the ability to make reliable quantitative estimates diminishes with each advance in our understanding of the mutational process. Nevertheless, the huge uncertainty surrounding estimates of the extent of radiation effects is being systematically divided into smaller areas of uncertainty which can be individually investigated. It is with the quantitative assessment of radiation-induced genetic damage that this paper is concerned, and attention is confined to damage incurred as a consequence of gonadal irradiation.

## Variation in Mutant Yield

Radiation-induced mutations are a motley group from whatever aspect they are considered. At one level they range from gross chromosome aberrations easily distinguishable under a microscope to a change in a single nucleotide pair within a DNA molecule. At a higher level, that of phenotypic effects, diversity is extreme, although it is unlikely that any radiation-induced mutations fail to duplicate those occurring in nature. This is a limitation of dubious value in view of the fact that there is probably a genetic component in most if not all human disabilities.

Essential to a reliable quantitative estimate of the genetic effects of radiation on man is an understanding of the complex relationship of mutant yield to exposure. It is precisely at this level that human data are most lacking. The radiation-induced rate of mutation is not known for any character in man and the great majority of his hereditary defects are in no way suited to individual observations of this kind. Eventually, it will be possible to make use of human cells grown in tissue culture. In the meantime, information on the factors affecting radiation-induced mutant yield must be gleaned from whatever source is available. Fortunately, it is at this basic level that information from other organisms is most likely to be useful; there is probably little radiobiological data from any source that cannot be applied in one way or another to human subjects, even if only to point out the problems which must be solved before reliable quantitative estimates can be obtained.

## Locus Specificity

Any attempt to estimate the amount of genetic damage induced by radiation must contend with the fact that there is variation between loci in the rate at which mutations are induced. This variation, first recognized in *Drosophila melanogaster* and plants, and later in microorganisms, has now been demonstrated in the induced rates of visible mutation at seven loci under study in mice (Russell and Russell, 1959). The highest and lowest of these differ by no less than a factor of 30, as shown in Table 1.

Other aspects of locus behavior are relevant. A gene can mutate to produce a range of different, if phenotypically related alleles with complex dominance relationships. Moreover, each is induced at a different rate. These facts have been well demonstrated in *Drosophila* (Timofeeff-Ressovsky, 1933), but they also hold for bacteria (Newcombe, 1952) and *Neurospora* (Giles, 1955). No basic understanding of such differences

TABLE 1—*Distribution of Mutations Found at Seven Specific Loci Following Irradiation of Spermatogonia in Mice at Various Doses*

| Locus | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| a | b | c | d | p | s | se | Total | Reference |
| 2 | 34° | 18†‡ | 25† | 22 | 71° | 2 | 174 | Russell and Russell (1961) |
| 0 | 2 | 0 | 3 | 0 | 0 | 5 | 10 | Carter et al. (1958) |
| Total 2 | 36 | 18 | 28 | 22 | 71 | 7 | 184 | |

° Four of these mutations occurred as two clusters of two each.
† Two of these mutations occurred as a cluster.
‡ Three of these mutations occurred as a cluster.

has yet been achieved, although a general pattern is discernible; more extreme phenotypic deviations appear more frequently than those of lesser effects (Muller, 1954). Data of Russell and Russell (1959) bear this out for the mouse; of 92 mutations at seven loci, 72 have been homozygous lethals. For the majority of these, death is delayed until the perinatal period, and in many instances until weaning.

Significant advances toward an understanding of the mutational process are being made through studies of the fine structure of genes (Demerec and Hartman, 1959; Benzer, 1961). Investigations of the RII region of the genome of phage T4 serve as an example. These have indicated the presence of hundreds of mutable sites within the gene or functional unit. The frequency of spontaneous mutation varies among these sites. Furthermore, the spectra of mutations induced by various mutagens differ from each other and from the spectrum of spontaneous mutations. An example of differences between the spectra of induced and spontaneous mutations is shown in Table 2. These are a selected group of mutations

TABLE 2—*The Frequencies of Spontaneous and 5-Bromouracil-Induced Mutations at Two Sites in the RII Region of Phage T4*
(Data from Benzer and Freese, 1958)

| | Total RII mutants | Site "A" | Site "B" |
|---|---|---|---|
| 5-bromouracil | 67 | 11 | 0 |
| Spontaneous | 126 | 0 | 19 |

which all produce the same ultimate phenotype. However, such studies will ultimately lead to an understanding of differences in mutation frequencies at different loci.

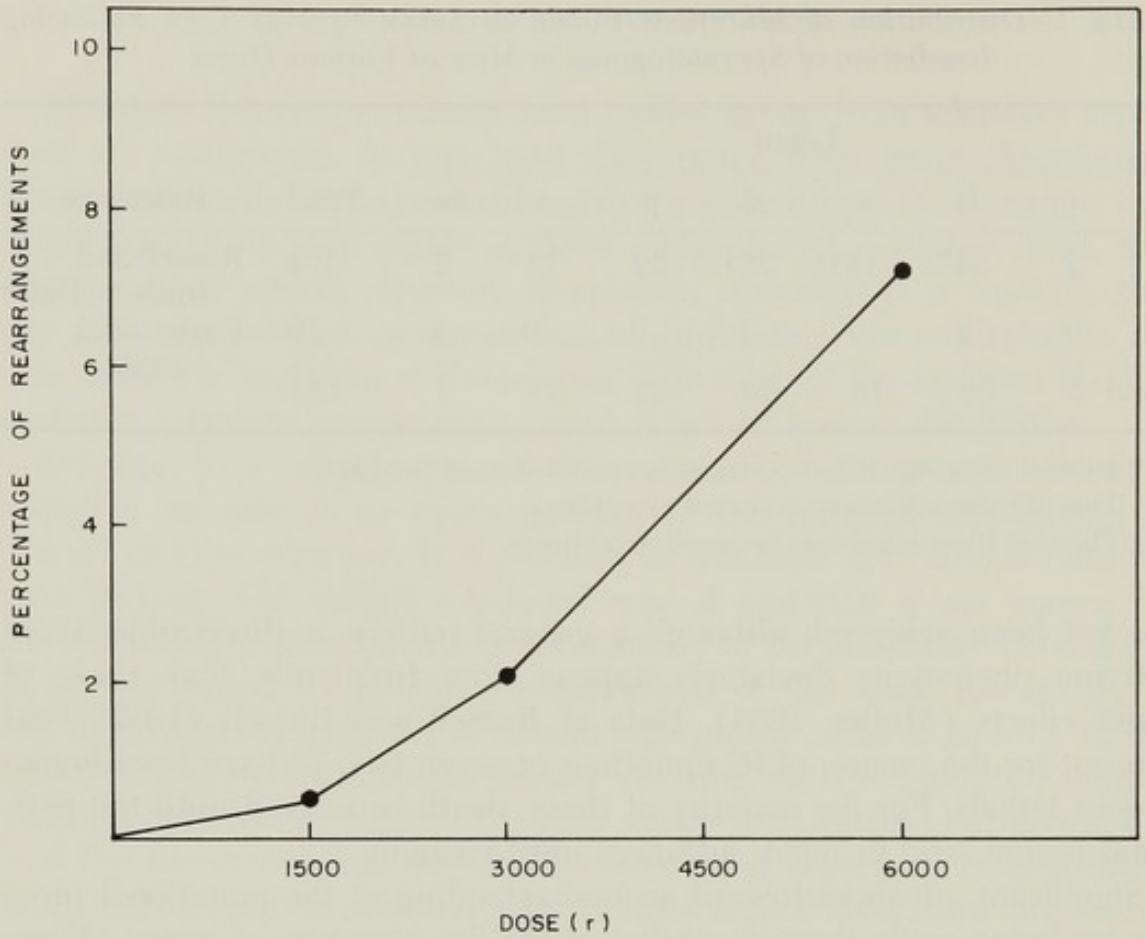


FIG. 1—Yield of viable types of chromosomal rearrangements induced by irradiation of spermatozoa of *Drosophila melanogaster* (from Timofeeff-Ressovsky, 1939).

## RADIATION DOSE

Experimental evidence of the linearity of dose-mutation curves early led to the conclusion that radiation-induced mutations are chemical changes which can occur as a consequence of a single ionization. The interpretation of biological effects in these terms has become known as the "target theory" (Lea, 1946). Importantly, this theory denies the existence of a threshold below which radiation is mutagenically ineffective and thus indicates that it is possible to predict the frequency with which mutations are induced at untested doses. Ability to make such predictions is particularly important when doses too low to permit experimental verification are concerned. The basic tenets of the target theory are supported by a large body of evidence, but perhaps the most rigorous confirmation has been provided by studies with bacterial viruses (Markovitch, 1956). Here, the mutagenic effect of low doses of radiation can be studied because it is possible to detect changes in the genetic apparatus of an infected bacterium even when these occur with very low frequency.

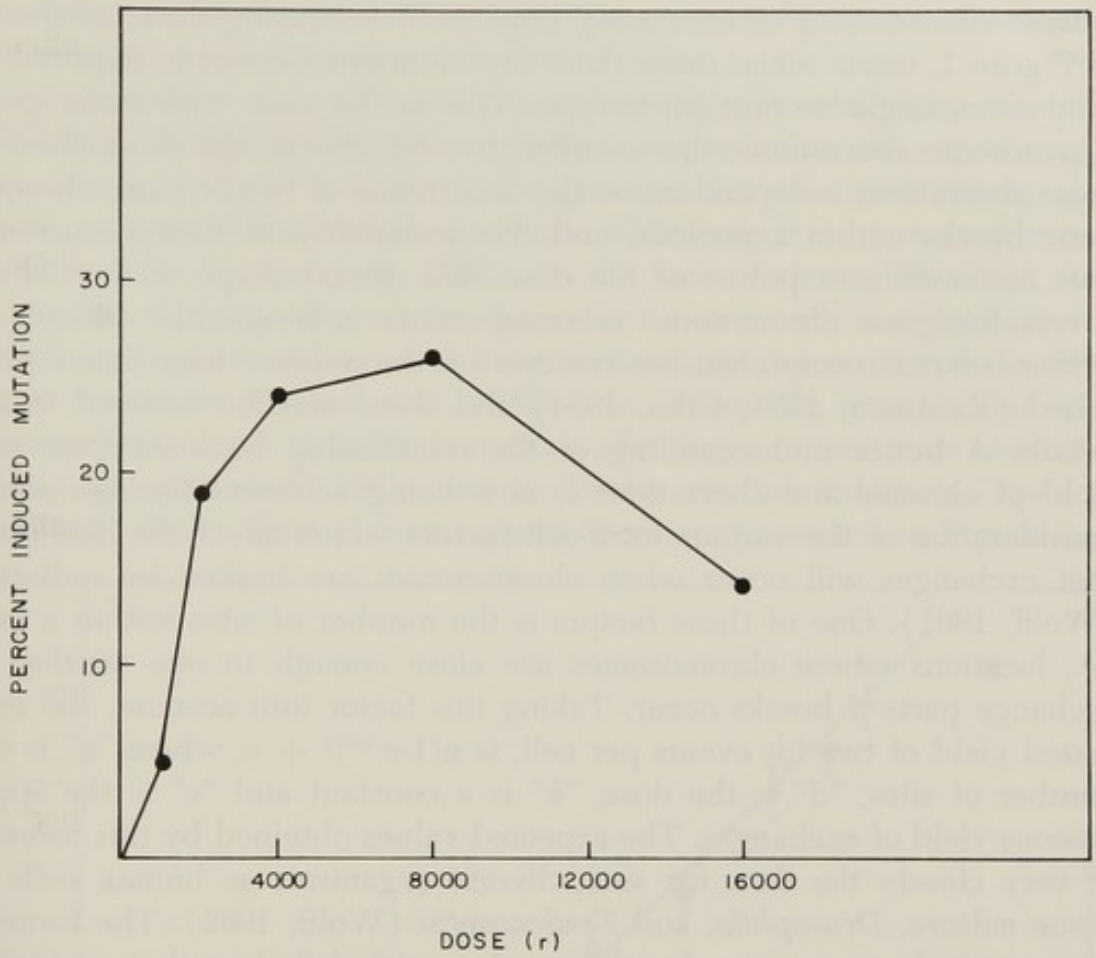


FIG. 2—Yield of mutations affecting colony morphology induced by x-irradiation of *Steptomyces* spores (from Newcombe and McGregor, 1954).

With exposures to less than one r, the mean number of ionizations per bacterium can be shown to be fewer than one. At such low doses the efficiency of radiation would be noticeably reduced if the genetic change required two ionizations. No such reduction has been noted; in fact, linearity of the dose-effect curve has been demonstrated over the entire range from 3,200r down to 0.3r.

Disconcertingly, the relationship of mutant yield to dose is not linear under many experimental conditions. Figures 1 and 2 illustrate contrasting experimental results. When nonlinearity occurs it is usually assumed that the yield of mutant phenotypes is not reflecting the original incidence of mutational events initiated by radiation. Frequently, it has been possible to confirm such an interpretation. However, in estimating genetic hazards of radiation it is the ultimate yield of mutations which assumes prime importance. Consequently, increased evidence of the validity of the target theory has by no means lessened the necessity for further studies of the dose-effect relationship.

Dose-effect curves of increasing slope with increasing dose, as shown in Figure 1, occur when more than one mutational event is required to produce a single mutant phenotype. This is the case with some gross chromosome aberrations, the so-called two-hit effects; the production of these aberrations is dependent on the occurrence of two or more chromosome breaks within a nucleus, and the probability of their occurrence thus increases as a power of the dose. The actual shape of dose-effect curves for gross chromosome rearrangements is frequently difficult to derive; several comprehensive reviews of the subject have been published (Kaufman, 1954; Giles, 1954) and these may be referred to for details. A better understanding of the relationship between dose and yield of chromosome aberrations is now being achieved through closer consideration of the various intra-cell factors which affect the likelihood that exchanges will occur when chromosomes are broken by radiation (Wolff, 1961). One of these factors is the number of sites within a cell, *i.e.*, locations where chromosomes are close enough to one another to exchange parts if breaks occur. Taking this factor into account, the estimated yield of two-hit events per cell, is $n(1\text{-}e^{-kd})^2 + c$, where "n" is the number of sites, "d" is the dose, "k" is a constant and "c" is the spontaneous yield of exchanges. The expected values obtained by this formula fit very closely the data for such diverse organisms as human cells in tissue culture, *Drosophila*, and *Tradescantia* (Wolff, 1962). The formula accounts in large measure for the usual empirical finding that, at higher doses, translocations increase as the 3/2 power of the dose.

Dose-effect curves for mutations frequently display a decreasing slope at higher dosages, as shown in Figure 2. This type of distortion has been attributed to a variety of causes. One is heterogeneity of the irradiated population of cells with regard to mutability or sensitivity. Another is that, at higher doses, the same locus may be hit more than once. A "saturation" effect also occurs when the same mutant phenotype is produced by mutations at different loci in the same cell. Demerec and Sams (1960) have attributed a drop in yield of mutations at higher doses to the fact that a mutation at one site of a multisite locus may be neutralized by the coincidental occurrence of mutation at another site in the same locus.

The existence of still other influences which distort dose-effect curves cannot be wholly excluded. Radiation might, for instance, modify non-genetic cellular processes which, in turn, influence one or another of the sequence of steps involved in the mutational event, and this could lead to distortion of the dose-effect curve for mutations.

Most of the known factors which distort the basic linearity of dose-effect curves are less effective at lower doses. Nevertheless, the potential genetic consequences of exposing large human populations to chronic

irradiation at low intensities has led to an increased interest in the exact nature of the mutation curve at the lower end.

Unfortunately, it is at lower doses that experimental data are most difficult to obtain. With higher organisms in particular, the experimental procedures are both arduous and expensive; for example, among over 60,000 progeny of male mice exposed to 37.5r, only 6 were mutant at any of seven loci (Carter et al., 1958). In the face of these difficulties, it is impressive that linearity of the dose-effect curve with low intensity irradiation of spermatogonia has been successfully demonstrated down to 86r in the mouse (Russell et al., 1958b). In *Drosophila*, linearity has been demonstrated down to 5r for mutations affecting a quantitative character (Glass and Ritterhoff, 1961) and to 25r for sex-linked lethals (Spencer and Stern, 1948). Data of Spencer and Stern are presented in Figure 3.

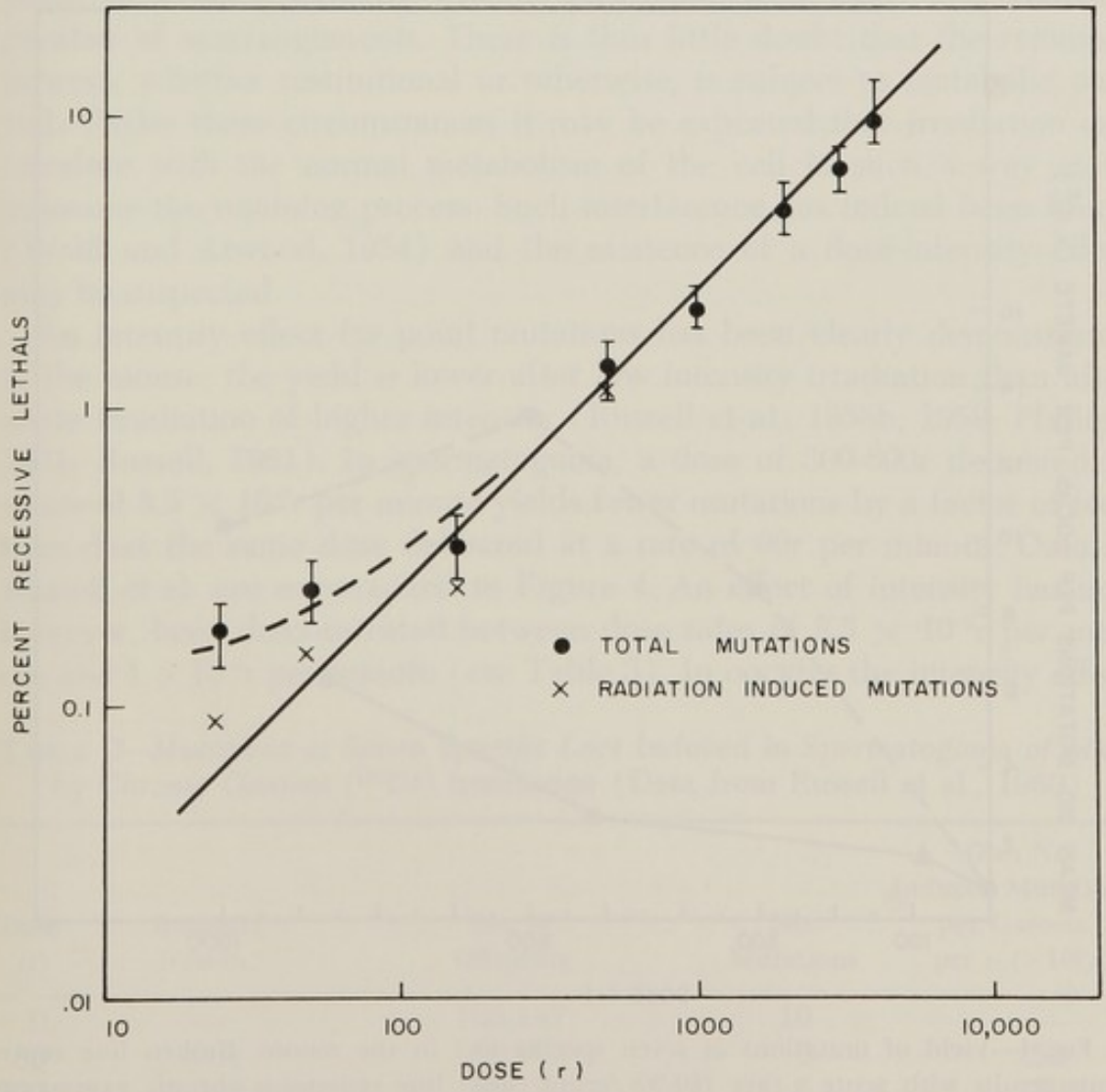


FIG. 3—Yield of x-ray-induced mutations in *Drosophila* at low doses. The values for induced mutations were obtained by subtraction of control values from the values for spontaneous plus induced mutations (data from Spencer and Stern, 1948).

## Radiation Intensity

The discovery that intensity of radiation can, under some circumstances, influence the yield of point mutations has been perhaps the most important of recent advances in radiation genetics. This phenomenon has provided new insight into the basic mechanisms of the mutation process; it has also complicated the problem of estimating the quantitative effects of irradiation.

Intensity effects for some types of gross chromosome aberrations have been recognized for many years (Sax, 1939). Here the effect has been attributed to the involvement of a time factor in the production of "multi-hit" aberrations: broken ends of chromosomes may 1) rejoin or "restitute"

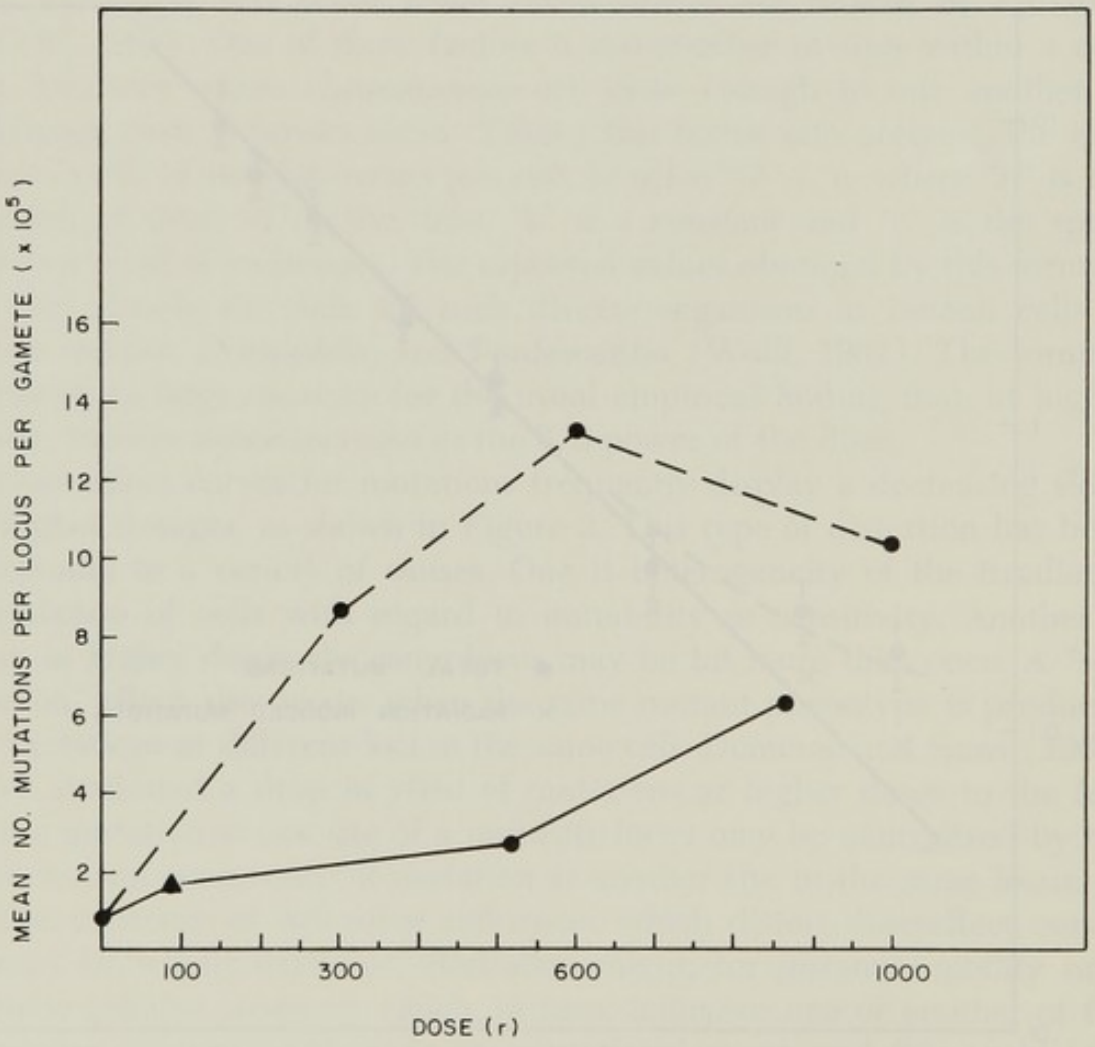


Fig. 4—Yield of mutations at seven specific loci in the mouse. Broken line represents results with acute x-rays (80-90r/min.). Solid line represents chronic gamma-ray results (triangle, 90r/wk; circles, 10r/wk). The value for zero dose represents the sum of all controls. Each point is based on data from more than 20,000 offspring of irradiated mice (from Russell et al., 1958b).

to reproduce the original state, 2) heal in the open position, an event which is possibly rare or absent in animals (Muller, 1954), or 3) rejoin with other broken chromosome ends in the same nucleus to produce an aberrant configuration. An initial chromosome break can lead to an aberrant configuration only if a second break occurs before restitution takes place. In consequence, the probability of restitution is greater with radiation of low intensity.

More recently another mechanism which may lead to an intensity effect for chromosome rearrangements has been observed. In plants, postirradiation treatments such as low temperature, cyanide, carbon monoxide, dinitrophenol, sodium hydrosulphite, and choramphenicol, have been found to inhibit rejoining, and therefore lead to an increased number of rearrangements, whereas treatment with exogenous adenosine triphosphate stimulates rejoining (Wolff, 1961) and thus leads to a reduced number of rearrangements. There is thus little doubt that the rejoining process, whether restitutional or otherwise, is subject to metabolic control. Under these circumstances it may be expected that irradiation can interfere with the normal metabolism of the cell in such a way as to influence the rejoining process. Such interference has indeed been found (Wolff and Atwood, 1954) and the existence of a dose-intensity effect may be suspected.

An intensity effect for point mutations has been clearly demonstrated in the mouse; the yield is lower after low intensity irradiation than after acute irradiation of higher intensity (Russell et al., 1958b, 1959; Phillips, 1961; Russell, 1961). In spermatogonia, a dose of 300-600r delivered at a rate of $8.5 \times 10^{-3}$r per minute yields fewer mutations by a factor of four than does the same dose delivered at a rate of 90r per minute. Data of Russell et al. are summarized in Figure 4. An effect of intensity has not, however, been demonstrated between dose rates of $8.5 \times 10^{-3}$r per minute and $1 \times 10^{-3}$r per minute (see Table 3). In oocytes the intensity effect

TABLE 3—*Mutations at Seven Specific Loci Induced in Spermatogonia of Mice by Chronic Gamma ($^{137}Cs$) Irradiation* (Data from Russell et al., 1960)

| Dose (r) | Intensity (r/min.) | No. of Offspring | No. Mutations | Mean No. Induced Mutations per Gamete per r ($\times 10^5$) |
|---|---|---|---|---|
| 0 | — | 166,147 | 10 | — |
| 86 | .001 | 56,993 | 6 | .52 |
| 300 | .009 | 28,170 | 5 | .39 |
| 516 | .009 | 26,321 | 5 | .25 |
| 861 | .009 | 24,281 | 12 | .50 |

is even more pronounced than in spermatogonia, and the range of effectiveness is greater.

Russell has interpreted this dose-intensity effect in terms of differences in amount of "repair" of premutational damage. It is assumed that radiation destroys a natural repair process at higher doses, as had been claimed earlier for restitution of chromosome breaks (Newcombe, 1942). The apparent absence of a dose-rate effect when intensities are lower than $8.5 \times 10^{-3}$r per minute suggests that a fraction of radiation-induced damage is irreparable. The fact that such natural repair systems are differentially affected by intensity of exposure has not yet been verified. However, the existence of natural repair of x-ray-induced damage leading to point mutation is undisputed. In *Paramaecium*, for example, the yield of point mutations is greater the nearer the cells are to duplication at the time of irradiation; this phenomenon is best interpreted in terms of a decreasing interval during which repair can occur (Kimball, 1961). Confirming this interpretation, delay of cell division after radiation exposure reduces the mutation yield, presumably by extending the period in which repair can take place.

The dose-rate effect in spermatogonia and oocytes of the mouse suggests the possible existence of such an effect in man, and recent estimates of genetic hazards of radiation have taken this into account (UN Report, 1962). The phenomenon is, however, by no means general and there is no certainty that it occurs in man. For instance, it has not been found in mature spermatozoa of either the mouse or *Drosophila*. It has been detected in the spermatogonia and oogonia of the silkworm (Tazima et al., 1961) but is absent, or weak, in the chalcid wasp *Dahlbominus* (Baldwin, 1962). In early germ-cell stages of *Drosophila*, initial indications of an intensity effect have, with accumulating data, become more doubtful (Oster et al., 1959; Purdom and McSheehy, 1961; Purdom, 1962; Muller et al., 1962).

## Dose Fractionation

The yield of induced mutations can be profoundly influenced by splitting the dose into fractions which are delivered at intervals. An understanding of this dose-fractionation effect is essential for predicting and minimizing the extent of genetic damage to be expected from medical exposures to radiation.

Fractionation is analagous to decreasing the intensity of a single exposure and might be expected to reduce mutant frequency. Reductions sometimes do occur. However, the procedure exposes any radiation-

induced modification of radiosensitivity, and not uncommonly the consequence of this altered sensitivity is an increase in mutant yield.

The total yield of chromosome rearrangements is often reduced with fractionation, because breaks produced during the initial exposure have an opportunity to restitute and are thus unavailable for exchange with those occurring during the subsequent exposure. Reductions attributable to such a mechanism have been well documented in plants (Bora, 1954). On the other hand, increases in frequency of chromosome aberration are not uncommon. For example, Sax (1961) has shown that a dose of 400r, delivered at the resting stage, can increase the radiosensitivity of chromosomes at a later prophase. The complexity of the factors affecting radiosensitivity to chromosome aberration has been succinctly described by Merz et al. (1961).

The yield of point mutations is similarly affected by a complexity of radiosensitivity changes accompanying irradiation. A protective effect of fractionation has been noted for somatic mutations in *Trifolium repens* (Davies and Wall, 1961). The extent of this protection first increases and then decreases as the interval between exposures is lengthened, and it has been hypothesized that induced mitotic delay and stage sensitivity are influencing factors. Investigations by Russell (1962) have recently revealed an opposite effect of dose fractionation on the yield of point mutations induced in spermatogonia of the mouse; the rate obtained with 1,000r delivered in two fractions separated by 24 hours exceeds by more than five times that observed with a single exposure to 1,000r. This increase cannot be explained in terms of cell selection. The length of the interval between exposures is crucial, but the most effective length is yet to be determined. Whether oocytes react similarly to dose fractionation, or whether the phenomenon exists at lower dose levels is not yet known.

## Cell-Stage and Type

The yield of mutations from a given exposure has been known for some time to vary with different tissues, strains and species. Cell selection pressure has been considered one of the major factors leading to these differences, and investigations continue to indicate that it does indeed frequently play a role. Nevertheless, improved experimental techniques and more intensive studies have emphasized that many other mechanisms are involved.

Variation in yield with mitotic and meiotic stage is best studied where synchronized cell divisions either occur naturally or can be induced. Perhaps the most precise measurements of variation in chromosome dam-

age have been made by Sparrow (1951) using *Trillium erectum*. He has demonstrated a fiftyfold variation in the induced rate during the course of cell division (see Fig. 5). The most resistant stage is interphase. Wolff (1961) has shown that much of such variation can be interpreted in terms of the number of sites within a nucleus where the chromosomes come close enough to each other to permit rejoining other than restitution. This number is affected by the amount of chromosome movement, by whether

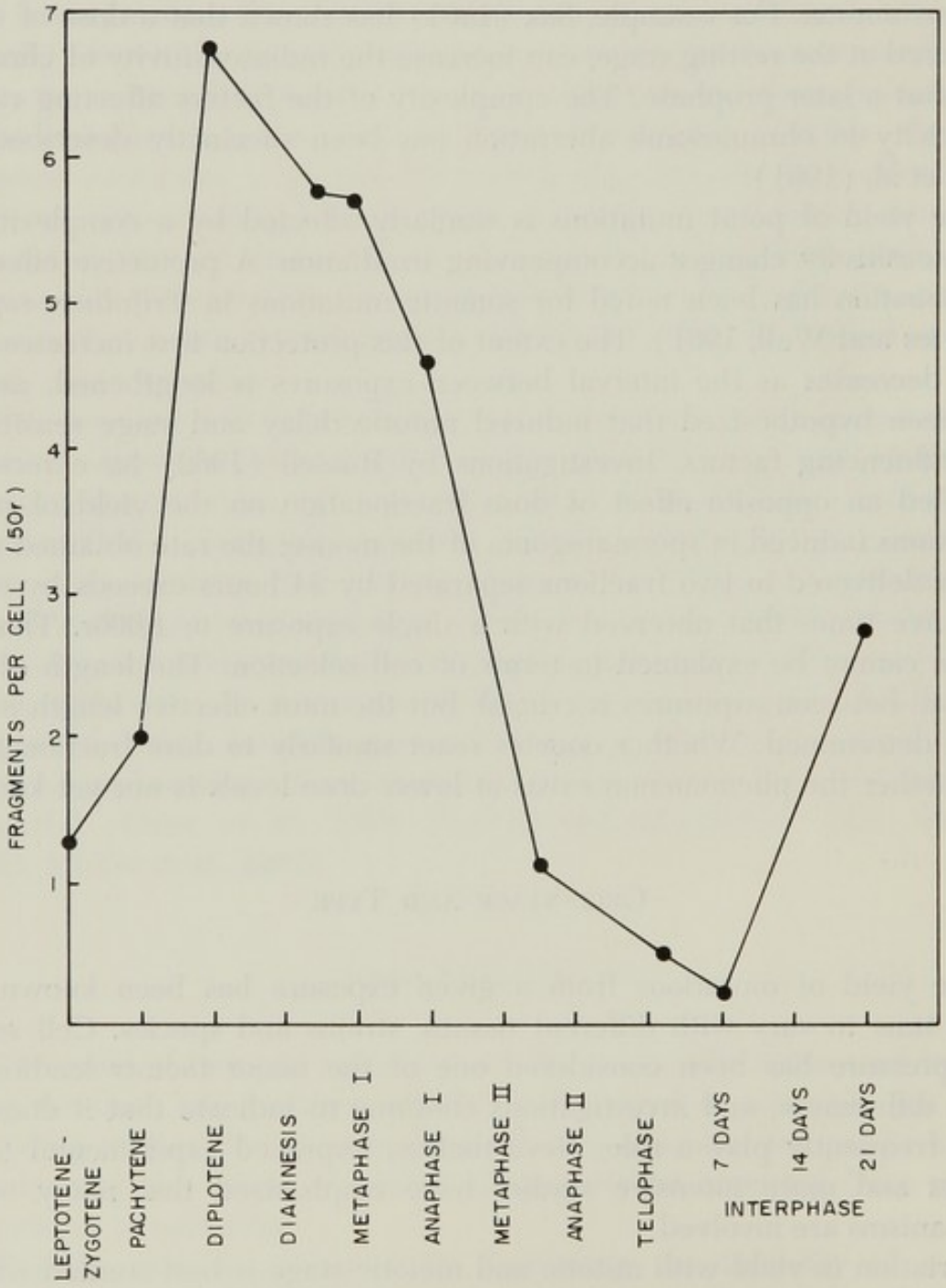


FIG. 5—Sensitivity to the induction of chromosome breaks by x-rays in different stages of cell division (from Sparrow, 1951).

or not the chromosomes have been duplicated at the time of irradiation, and presumably by other factors as well. Respiration rate, for instance, is reduced in dividing cells as a result of irradiation, and this may lead to an increased oxygen content with a consequent increase in radiosensitivity (Evans, 1962).

The yield of point mutation also varies with mitotic stage. Variation in *Paramaecium* (Kimball, et al., 1959; Kimball, 1961), has been mentioned; in the postduplication interphase and early prophase of this organism no more than 10 per cent of the maximum mutation rate can be induced. The general pattern of variation during the cell cycle has been confirmed in such widely separated organisms as *Habrobracon*, *Drosophila* and mice.

The influence of stage of gametogenesis on mutation in mammals is of particular interest because it bears directly on radiation hazards. A difference in the frequency with which chromosome aberrations are induced in spermatogonia and postspermatogonial stages has been recognized for many years; the yield is much higher with irradiation of postspermatogonial stages. The subject has been comprehensively reviewed by W. L. Russell (1954) and by L. B. Russell (1962). The induction of aneuploidy in mice has received less attention, but studies are now being made at Oak Ridge. These have shown that the interval between sperm penetration and first cleavage is particularly sensitive in the production of chromosome loss (Russell and Saylors, 1960). Irradiation of spermatozoa has also been shown to be effective in producing loss of a paternal sex chromosome (Russell and Saylors, 1962).

The frequency with which point mutations are induced is also lower in spermatogonial stages than that in postspermatogonial stages. In the mouse the difference is twofold (Russell et al., 1958a). Furthermore, there is less variation in the rates at which specific loci mutate, the range being about threefold for spermatogonial stages as compared with thirtyfold for postspermatogonial stages.

In the female, the stage of the oocyte has a profound effect on the frequency with which dominant lethals are induced; as might be expected, sensitivity is greatest in early meiosis (see Table 4). The induced rate of point mutation, at least with acute irradiation, is higher in oocytes than in spermatogonia (Russell et al., 1959).

The fact that species differ in radiosensitivity has been emphasized by the demonstration that the mouse is apparently about fifteen times as sensitive as *Drosophila* (Russell, 1956). Variation also exists between strains within a species, as has been demonstrated for *Drosophila* (Dubovsky, 1935) and more recently, for mice (Searle, 1961; Froelen, 1962). Oakberg and Clark (1962) have shown that differences in the meiotic

TABLE 4—*Frequency of Dominant Lethals Induced in Oocytes by Irradiation (400r) at Various Intervals prior to Fertilization*
(From Russell and Russell, 1956)

| Mean Treatment-to-Fertilization Interval (hr.) | No. Corpora Lutea | No. Living Embryos | Freq. Dominant Lethals* |
|---|---|---|---|
| 16.1 | 42 | 31 | .19 |
| 12.6 | 84 | 36 | .53 |
| 8.5 | 92† | 1 | .99 |
| Controls | 119 | 108 | — |

* $1 - \frac{\text{living embryos/corpora lutea (for irradiated females)}}{\text{living embryos/corpora lutea (for control females)}}$

† To compute dominant lethals in this group an average number of corpora lutea equal to controls was assumed because corpus luteum counts are not reliable when few or no living embryos are present.

prophase stage at which oocyte development is arrested could be one important factor leading to differences in mammalian response to radiation.

Despite the obvious complexity of the factors leading to observed differences in mutant yield between cells and between organisms, it seems likely that extrapolation to human subjects will eventually be feasible. Advances may, for example, be expected through comparisons of mammalian and human cells grown *in vitro*. Research along these lines is relatively new, and is currently directed mainly toward perfecting techniques and toward devising methods and testing their application to extrapolations between multicellular organisms. Many influencing factors remain to be understood and controlled. For instance, rates of spontaneous mutation increase with the length of time cells have been in culture (Sax and Passano, 1961), and rates of both spontaneous and induced mutation vary with cultural conditions. Cytologically demonstrable chromosome aberrations in human cells cultured *in vitro*, have been studied by a number of investigators (Bender, 1957; Puck, 1958; Chu et al., 1961; Lindsten, 1959). The sensitivity of human epitheliod cells apparently does not differ greatly from that of monkey epitheliod cells and the fibroblast cells of Chinese hamsters (Bender and Wolff, 1961). Extrapolating also from tissue culture to whole animals, the coefficients of aberration production for chromatid breaks in epitheliod-type cells *in vitro* and for chromosome breaks in leukocytes of freshly drawn blood are in remark-

ably good agreement with those for *Tradescantia* microspores (Bender and Gooch, 1962).

Of particular significance to the problem of extrapolation is the work of Sparrow and Evans who have been able to predict radiosensitivity of different species of plants with considerable accuracy from a knowledge of nuclear volumes and chromosome numbers (Sparrow and Evans, 1961; Evans and Sparrow, 1961). Pertinent data are presented in Table 5. In

TABLE 5—*The Relationship between Chromosome Number, Nuclear Volume, and Radioresistance in Plants (Data from Sparrow and Miksche, 1961)*

| Species | Chromosome No. | Av. Vol. of Root-Tip Nuclei ($\mu^3$) | Nuclear Vol. per Chromosome | Radio-Resistance* (r/day) |
|---|---|---|---|---|
| *Glycine max* | 40 | 150 | 3.8 | 400 |
| *Zea mays* | 20 | 280 | 14.0 | 375† |
| *Allium cepa* | 16 | 570 | 35.6 | 150 |
| *Vicia faba* | 12 | 490 | 40.8 | 120 |
| *Lilium henryi* | 24 | 1,100 | 45.8 | 80‡ |
| *Tradescantia paludosa* | 12 | 640 | 53.3 | 40 |

* Daily dose required to produce severe growth inhibition (in gamma greenhouse).

† Estimated value based on field experiment.

‡ Estimated value based on data for *Lilium longiflorum*.

these studies radiosensitivity is estimated in terms of growth inhibition which has been related to chromosome damage (Read, 1959). Sparrow and Evans have shown that nuclear volume is related to DNA content, and that the average DNA content per diploid nucleus or per chromosome is highly correlated with radiosensitivity. Furthermore, an increase in chromosome number without a change in nuclear volume has a protective effect. Other nuclear and allied factors which influence radiosensitivity have been listed (Evans and Sparrow, 1961). There is also evidence that the frequency of somatic mutation (marker loss) per r is related to nuclear size (Sparrow and Evans, 1961).

## External Factors

The fact that radiation can induce mutations indirectly through its effect on water or other cell constituents, and that mutation need not be an instantaneous chemical change but rather the consequence of a prolonged sequence of changes, has led to a search for treatments that can

influence the yield of mutation. This search has been successful and many effective treatments are now known. These may precede, accompany, or follow irradiation. Pretreatments are usually related to the oxygen effect, a reduction in genetic damage when irradiations are performed anoxically. The oxygen effect is usually attributed to the production of a damaging compound such as hydrogen peroxide. Post-treatments are usually considered to influence some natural repair system. These can seldom be considered independently of pretreatment or accompanying treatment; either of the latter may alter the ability of a cell to respond to post-treatment, and treatments during irradiation may well involve repair mechanisms. Post-treatments are usually carried out with agents whose biological actions are well known, and attempts are made to interpret the results in terms of these known actions. However, side effects of such agents may also be involved. The subject of recovery and protection of cells from radiation-induced genetic injury has been so adequately covered in recent publications that it will not be considered further here (Conger, 1960; Wolff, 1960).

## Estimates of Mutant Yield

The rarity of individual mutations, the variation between loci in mutation rate, and uncertainty about the effective number of loci, combine to limit the reliability of surveys of individual loci in providing a representative estimate of induced mutant yield. Consequently, it is more usual to consider mutation in terms of the yield per gamete or chromosome and to classify phenotypic effects into meaningful, if loose, groups. These groups frequently overlap to some extent, and as will be evident from the text which follows, much of the classification is a natural consequence of the experimental procedures used to expose the mutations. The classes most frequently considered are 1) dominant lethals, 2) dominant visibles, 3) sex-linked recessives, 4) autosomal recessives, and 5) subvitals and supervitals.

These classes can be assessed individually for the effect of an increase in mutation rate on the well-being of a population. However, in practice only a few of them are amenable to direct study in man and these do not necessarily include those mutations which constitute a more important genetic hazard. For this reason each class is more suitably investigated as an indicator of over-all genetic damage.

The usefulness of any class of mutations as an over-all indicator of induced mutation rates is hampered by the fact that the rates undoubtedly differ for different classes. For instance, a study of induced mutation

rates in the mouse (Lyon et al., 1962) has indicated that the rate of induction of recessive lethals is nearly twice that of dominant lethals, whereas the rate for recessive visibles is about one-fifth that of dominant lethals. The same study detected too few dominant visibles to warrant the calculation of a rate. It is technically possible to circumvent this difficulty, at least partially, by relating induced mutant yield to spontaneous yield. This relationship is usually defined in terms of the "doubling dose," the radiation exposure which induces the same number of mutations as occur spontaneously in one generation. The doubling dose can be expected to provide a key for extending information from one class of mutation to another. It is not illogical to assume that the doubling dose is the same for many classes of mutation, at least under specifically defined circumstances of irradiation, or, alternatively, that a representative doubling dose can be determined for mutational damage considered collectively. Thus, once a usable doubling dose has been established, the burden of investigation is shifted from the study of induced mutation to the study of spontaneous mutation and the magnitude of its consequences. In man, it is now feasible, if difficult, to estimate spontaneous rates for some specific defects with a reasonable degree of accuracy (Crow, 1961; Penrose, 1961), and continued studies of the genetic structures of human populations and the selective pressures to which these populations are exposed can be expected to provide reliable data on spontaneous mutation within whole classes.

There is no simple relationship between the spontaneous mutability of individual gene loci and their sensitivity to radiation-induced changes. In fact, early investigations in maize indicated a qualitative difference, mutations due to ionizing radiation being confined to losses of genetic material (Stadler, 1941). Later investigations, summarized by Muller (1955) have suggested that maize is exceptional in this respect. Nevertheless, studies with lower organisms have demonstrated that radiation does not increase the likelihood of mutation at all loci by the same factor (Newcombe, 1952; Giles, 1955; Demerec and Sams, 1960). This circumstance does not disallow the calculation of a representative doubling dose for a class of mutations provided one is willing to forego information on the change in the spectrum of mutations which would accompany an increased rate. The usefulness of the doubling dose is more severely limited by the profound influence of such factors as radiation intensity and cell stage on the yield of mutations. It is evident that the doubling dose for acute irradiation varies with different conditions of exposure, and that these conditions must be carefully defined if accurate estimates are to be obtained.

Experimental organisms are being used with increasing frequency to

provide quantitative estimates of mutant yield. Although these investigations are usually undertaken in the interests of man and not of the organism under test, some uncertainty exists as to how they can best serve this purpose. There is reason to suppose that some quantitative data from higher organisms might be directly extrapolated to man, as a guide at least. In the absence of a more suitable organism, the mouse is now used extensively for this purpose. However, suspected differences in the effective number of loci, in the frequency with which mutations are induced, and a lack of strict comparability of phenotypic classes often render direct extrapolation suspect. However, at the very least, quantitative data in such organisms can be usefully applied in testing the validity of doubling-dose concepts.

An observed rough similarity of doubling doses in different organisms warrants consideration. The relatively long life span of man suggests that his spontaneous mutation rate per generation may be higher than that of shorter lived organisms. If so, and assuming a similarity between species in radiation sensitivity of genes, a higher doubling dose is to be expected for man. Nevertheless, estimates of doubling doses based on specific conditions in mammals, insects and plants, have shown remarkable similarity (Waddington and Carter, 1956), and it may well prove that these values, at least in mammals, are more directly useful than now seems likely.

## Dominant Lethals

Any mutation which causes the death of an individual heterozygous for that mutation is, by definition, dominant lethal. With higher organisms the time at which the induced death occurs is variable and depends on both the characteristics of the mutation and on external factors. At one extreme, death may take place in the germ line; at the other, it may occur so late in life that progeny have already been produced. The class of mutations so defined is, in general, too inclusive to be useful, and investigations usually restrict the class by stipulating a specific period during which death occurs. In mammals, a lethal is usually defined as a mutation which causes death of the embryo prior to birth.

Dominant lethals have received much attention both as a potential hazard of radiation and as a possible indicator of over-all genetic damage. Two properties of the class have invited this attention: the mutants can be induced with great frequency in experimental animals, and they can be detected in the immediate progeny of irradiated parents.

A method considered promising for the detection of radiation-induced

dominant lethals in man involves the observation of a shift in the sex ratio of the progeny of irradiated males. A change in the sex ratio is to be expected from the fact that daughters receive dominant lethals via both the X-chromosomes and the autosomes. Sons, on the other hand, can receive them only via the autosomes, providing the Y-chromosome is inert. The excess of dominant lethals in daughters is projected as an increase in the proportion of male progeny.

The results of five relevant studies have been reported (Macht and Lawrence, 1955; Schull and Neel, 1958; Tanaka and Ohkura, 1958; Lejeune et al., 1960; Scholte, 1962). The data have been drawn from various sources and are summarized in Table 6. Results have been disconcert-

TABLE 6—*The Effect of Paternal Irradiation on the Proportion of Male Offspring*

| | Control | | Irradiated | | |
|---|---|---|---|---|---|
| Study | No. Live Births | % Male | Dose (r) | No. Live Births | % Male |
| Macht and Lawrence (1955) | 3,491 | 52.4 | unknown | 4,277 | 51.4* |
| Schull and Neel (1958) | 43,544 | 52.1 | ca.8 | 5,168 | 51.6* |
| | | | ca.60 | 1,226 | 53.3 |
| | | | ca.200 | 753 | 52.7 |
| Tanaka and Ohkura (1958) | 897 | 52.6 | 10-100 | 659 | 55.5 |
| Lejeune et al. (1960) | 1,185 | 51.5 | 200-400 | 656 | 56.1 |
| | 1,926 | 52.7 | 2-20 | 1,394 | 46.0* |
| Scholte (1962) | 828 | 46.6 | 300-600 | 635 | 52.3 |
| | 657 | 52.3 | 1-10 | 668 | 53.4 |

* Difference in the direction opposite to expected.

ingly variable; some have demonstrated a significant shift in the direction predicted by the genetic theory advanced above, but others have demonstrated a significant shift in the opposite direction. Furthermore, control values are sometimes erratic. It is, of course, possible to provide explanations of these results that do not conflict with known genetic principles. But whatever the cause or causes of the inconsistencies, it is evident that sex-ratio changes in the offspring of irradiated males do not at present provide a reliable index of radiation-induced dominant lethals in the X-chromosome. Confirming this is the fact that no consistent effect of radiation has been detected in comparable experiments with mice (Hertwig, 1938; Russell, 1954; Kohn, 1960).

In mammals, the induced yield of dominant lethals is most simply obtained by comparing the mean litter sizes of treated and untreated groups. Intra-uterine selection pressures undoubtedly lead to inaccura-

cies. With irradiated females inaccuracies are probably compounded by the influence of the irradiated maternal environment. Reliability of data is improved by counting live and dead embryos, implantation sites, and corpora lutea, in pregnant females.

Cell stage at the time of irradiation is particularly influential in determining yield of dominant lethals. Irradiation of later germinal stages in the male results in a drastic reduction in litter size attributable to the induction of dominant lethals. One of the largest of several investigations of rate of induction of dominant lethals was carried out by Russell and co-workers. The survey detected a frequency of 35 per cent in the progeny of 345 litters with a dose of 600r (Russell, 1957). Irradiation of spermatogonia yields a lower frequency of induced dominant lethals; thus Russell (1954) observed an incidence of only 3 per cent after a dose of 600r, and Carter and Lyon (1961) observed a similar incidence after doses of 300 and 600r. More recently, however, Lyon et al. (1962), detected an increase of 15 per cent with a total dose of 1,200r delivered in two fractions separated by an interval of 8 weeks. With irradiation of females (oocytes) Russell and Russell (1956) have succeeded in showing that a high proportion of embryonic deaths is a consequence of the induction of dominant lethals. The frequency with a dose of 400r varies from 19 to 99 per cent depending upon which stage in oogenesis is irradiated (see Table 4).

Cytogenetic studies have shown that the majority of radiation-induced dominant lethals are a consequence of gross chromosome aberration. The role of chromosome aberration has been studied most extensively in insects and plants, but the observed frequency in the mouse is usually sufficient to account for the deaths.

## Dominant Visible Mutations

A mutation is regarded as belonging in the category of dominant visible, in the strict sense, only if its phenotypic expression is similar in the homozygous and heterozygous states. However, the individual frequencies of "visible" mutations, or at least of those producing a degree of detriment, are so low in human populations that they rarely occur in the homozygous condition and their expression in this state can only be conjectured. Consequently, the class is more loosely and usefully defined to include any mutation which is conspicuously expressed in the heterozygote at birth or later. For practical reasons, studies are usually restricted to traits that are defective and observable at birth or shortly thereafter.

As a tool for the investigation of radiation effects, dominant visibles have many of the attributes of dominant lethals; most importantly the mutations can be detected in the progeny of irradiated individuals except where penetrance is reduced. Despite these advantages, they are of dubious value as an indicator of over-all genetic damage. The class lacks the characteristic of being sharply defined, and such factors as efficiency of diagnostic procedures introduce uncertainties. Perhaps the most serious disadvantage of the class is the fact that there is a good deal of ignorance about which of those defective traits arising in a population are, in fact, a consequence of dominant mutation. Many of the individuals suffering from conspicuous defects fail to produce progeny for one reason or another, a circumstance which precludes direct attribution of the traits to dominant mutation; a United Nations report (1958) lists for man only 57 men now clearly recognized as such. These are far too few to serve as a basis for estimating induced mutation rates. Furthermore, most of these are not recognizable at birth.

With advances in human cytogenetics, it has been possible to attribute a proportion of visible defects to gross chromosome aberration, including translocation. Current information has been reviewed by Ferguson-Smith (1961). Spontaneous and induced mutation to these defects are particularly amenable to study, and much relevant information can be expected to appear in the near future. One of these defects, Down's syndrome, has been studied with regard to induced mutation rate: a survey carried out by Uchida and Curtis (1961) noted a possible increase in frequency with maternal irradiation. Two others were completely negative (Carter et al., 1961; Stevenson and Matousek, 1962).

The rate at which dominant visible mutations are induced by radiation is readily determined in many experimental organisms. Unfortunately, the practical usefulness of precise quantitative interspecific comparisons of dominant defects is doubtful; the estimated rates depend heavily upon the anatomical and physiological criteria of the observations, and these are likely to be very different for different species. For this reason few serious attempts have been made to obtain quantitative data on dominant visibles in mammals. No doubling doses have been estimated. Surveys in organisms other than man have however, shown that visibles are induced with a low frequency compared to other types. Thus, Russell (1951) found only five dominant visible mutations among 30,000 offspring of male mice exposed to 600r. The induction of translocations has been considered a matter of minor importance at least with spermatogonial irradiation. However, investigations of Searle (1962) have indicated that a dose of 1,200r to spermatogonia in mice induces translocation in about 5 per cent of gametes.

## Sex-Linked Recessives

Mutations in man which, as a class, have received the most attention as indicators of radiation-induced genetic damage are sex-linked recessives; they not only form a discrete group but can also be detected in the immediate progeny of irradiated individuals. Located on the X-chromosome, they are expressed in the male progeny of irradiated females, it being assumed that the Y-chromosome is relatively inert.

The number of specific defective traits in man attributable to sex-linked genes is far too low to be of practical use in induced mutation studies; a United Nations Report (1958) lists only eight defective traits which have been so classified with any degree of assurance. Sex-linked recessive lethals, on the other hand, can presumably be induced at any one of many loci and are thus expected to form a class which is large enough to be of practical utility.

With maternal irradiation, sex-linked lethals that are expressed prior to birth are revealed by a reduction in the proportion of male progeny. The reliability of estimates obtained by this procedure has been seriously questioned on the grounds that many extraneous factors can influence the sex ratio. For instance, induced dominant autosomal mutations may have a different degree of expression in males and females, and there may be a differential effect of the irradiated intra-uterine environment on embryos of the two sexes. There is, moreover, a perplexing variation in the proportion of male offspring in control populations.

Nevertheless, surveys of the effects of maternal irradiation on sex ratio have been more consistent in their findings than have those dealing with paternal irradiation. To date the results of three surveys have been published (Schull and Neel, 1958; Lejeune et al., 1960; Scholte, 1962). These are summarized in Table 7. By far the largest of these investigations was that of Schull and Neel. It indicated that the induced mutation rate is $6 \times 10^{-5}$/maternal X-chromosome/rep. That of Lejeune et al., indicated a similar rate. The data of Scholte, as summarized, are insufficient to provide a value.

An opportunity to estimate spontaneous mutation rates to sex-linked lethality is provided by changes in the sex ratio with aging of the mother (Lejeune and Turpin, 1957). However, such a procedure must take into account an effect of birth order and paternal age (Novitski and Kimball, 1958). A reliable doubling dose has yet to be obtained.

More reliable, if less direct, estimates of radiation-induced sex-linked recessive lethal mutations can be obtained through surveys of the progeny of daughters of irradiated individuals. Those daughters which contain a sex-linked lethal should produce only half the expected number of male

TABLE 7—*Effect of Maternal Irradiation on Proportion of Male Offspring*

| Study | Control | | Irradiated | | |
|---|---|---|---|---|---|
| | No. Live Births | % Male | Dose (r) | No. Live Births | % Male |
| Schull and Neel (1958) | 43,544 | 52.1 | ca. 8 | 19,610 | 52.0 |
| | | | ca. 75 | 3,958 | 51.4 |
| | | | ca. 200 | 2,268 | 51.2 |
| Lejeune et al. (1959) | 355 | 54.6 | 200-400 | 161 | 44.7 |
| | 674 | 50.1 | 2-10 | 797 | 52.2 |
| Scholte (1962) | 225 | 53.3 | 300-600 | 221 | 48.0 |

progeny. The method has not yet been used in human subjects, but Cavalli-Sforza (1962) has used the procedure to estimate the rate of spontaneous sex-linked recessive lethal mutation in man. The average value for both sexes, admittedly tentative, is 0.017 per generation. From his results he has calculated that, using geographical areas where the doubling dose has been reached, it would be possible to estimate induced mutation rates with a population of 100,000 individuals.

For mice there is not much data on the induced rate to sex-linked lethality. However, Sugahara et al. (1961) have observed a shift in sex ratio, although nonsignificant, after chronic irradiation through three successive generations. The calculated mutation rate was $2.8 \times 10^{-3}$/X-chromosome/r. Searle (1962) has used the method of Cavalli-Sforza. He has calculated that with spermatogonial irradiation the frequency of recessive lethal gene or chromosome mutations is $1.7 \times 10^{-4}$/X-chromosome/r with high intensity irradiation. The spontaneous mutation rate is not yet known and so no doubling dose can be estimated.

## AUTOSOMAL RECESSIVES

Autosomal recessive mutation rates, whether induced or spontaneous, can be estimated only by indirect methods in higher organisms. In man, it is possible to devise practical procedures for estimating spontaneous rates but attempts to estimate induced rates are most impractical.

A method has recently been devised for estimating spontaneous mutation rates for recessives as a class (Morton et al., 1956; Morton, 1960). The procedure utilizes prevalence of recessive alleles and their mean selective disadvantage in the heterozygote. The prevalence of recessives

is estimated from information on the increase in death or disability among the progeny of consanguineous marriages and from a knowledge of the extent of inbreeding in the population. Recessive genes are considered in terms of equivalents. A lethal equivalent has been defined by Morton et al. as "a group of mutant genes of such number that, if dispersed in different individuals, they would cause on the average one death, *e.g.*, one lethal mutant, or two mutants each with 50 per cent probability of causing death, etc." In a corresponding manner, viable recessive defects can be defined in terms of detrimental equivalents.

Several surveys have been concerned with estimating the mean number of lethal and detrimental equivalents in human populations. The results of these have been summarized by Newcombe (1962). The studies have not been consistent in their scope and the estimates derived from some of them are probably not reliable for one reason or another. Nevertheless they suggest that individuals normally carry 2 to 4 lethal equivalents which, in the homozygote, are expressed as death prior to the age of 30. Estimates of the number of detrimental equivalents are similar. At present, for estimates of the dominance of these genes (expressed as genetic fitness) it is necessary to rely on *Drosophila* data. Several surveys of spontaneous and radiation-induced recessive lethals have indicated a mean reduction of about 2 per cent for preadult viability (Stern et al., 1952; Cordiero, 1952; deCunha et al., 1958; Goldschmidt and Falk, 1959; Hiraizumi and Crow, 1960). No value for less detrimental mutations has been estimated. With the above value it is possible to calculate a spontaneous mutation rate. With 4 lethal equivalents per gamete this turns out to be .08 per gamete per generation (Morton et al., 1956).

The spontaneous mutation rates to specific recessive defects in man have been estimated in a number of instances. Methods and current information have recently been reviewed by Crow (1961) and by Penrose (1961). The values are not considered to be very reliable nor are the loci concerned thought to be representative. Nevertheless, the estimates center around $2 \times 10^{-5}$/locus/generation.

In experimental mammals, induced mutation rates can be estimated with a considerable degree of efficiency by using various mating schemes. Rates can be determined over the entire genome or at specific loci.

One procedure for estimating recessive mutation rates over the entire genome involves the outcrossing of $F_1$ males (presumed heterozygous for recessive autosomal mutations) to normal animals and the subsequent back-crossing of their daughters to them. With mice Carter (1957) has obtained data indicating a rate of $3.3 \times 10^{-3}$/gamete/r for recessive lethals. In a later investigation Carter and Lyon (1961) were unable to estimate the rate of induction of recessive lethals because of the obvious

existence of intra-uterine compensation. However, a modified repeat of this experiment (Lyon et al., 1962) provided an estimate of $2 \times 10^{-4}$/ gamete/r a value which is about 1,000 times the mean rate for seven specific loci.

A second method of measuring induced autosomal recessive lethals in mice has been proposed by Haldane (1956). This involves the use of marker genes and has been used by Carter (1959) and by Sugahara et al. (1961). Data of Carter indicated that the induced rate was less than $1.2 \times 10^{-3}$/gamete/r. This value is considered by Haldane (1960) to be an underestimate. The data of Sugahara et al., implied rates of $4.3 \times 10^{-3}$/ gamete/r and $6.7 \times 10^{-3}$/gamete/r.

No data on spontaneous mutation rates to recessive lethality in the mouse have been published, so the doubling dose is unknown.

The specific locus method has been used in the mouse to determine the mean induced rate of recessive visible mutation at seven loci. Rates have been obtained for acute and chronic exposures of spermatogonia and oocytes. The estimates are undoubtedly highly accurate since they are based on data obtained from over one million progeny of irradiated and control animals. The mean value following acute spermatogonial irradiation of 300r is $2.8 \times 10^{-7}$/locus/gamete/r (Russell et al., 1958c). A doubling dose of about 30r can be calculated. Unfortunately, there is little reason to suppose that the loci under test are a representative sample and considerable reason to suppose that they are not; they were selected for study as loci known to mutate and to produce readily identifiable phenotypes. Furthermore, the great variation in induced rates among them suggests that the number of loci sampled is too small. Russell and others have repeatedly emphasized the unreliability of the values as estimates of over-all mutation rates. Nevertheless, they are extensively used for just this purpose for the simple reason that no better values are available. They are also used frequently to gauge the reliability of induced mutation rates obtained by other methods.

## Subvitals and Supervitals

Mutations which are neither lethal nor otherwise conspicuous in the homozygous state form a large class about which, nevertheless, very little is known. Not only are they difficult to detect, but their expression is often subject to modification by the external environment and the genetic background.

Probably any character is subject to modification by "minor" mutations, but investigators find it expedient to utilize those which are affected by

mutations at many loci. Typical examples are intelligence, birthweight and height. Such characters are termed quantitative and the mutations of small effect, polygenic. Studies of mutations affecting quantitative characters are often enlightening with respect to particular aspects of the genetic hazard of radiation. However, it is more usual to consider minor mutations in terms of their effect on such characters as fertility or preadult viability. These bear a relation to genetic fitness so that it is possible to predict the amount of damage that will be transmitted to subsequent generations and to make quantitative comparisons with lethal mutations.

Although minor mutations have a degree of expression in the heterozygous state and can be so investigated, they can be distinguished from lethals and other more detrimental mutations only by isolating them in the homozygous state. Relatively few investigations have made this attempt and these, for technical reasons, have been confined to lower organisms. It has been found that, in general, the degree of severity is inversely correlated with frequency of induction (Timofeeff-Ressovsky, 1935; Kerkis, 1938; Käfer, 1952; Falk, 1955; Bonnier and Johnson, 1957; James, 1959). Figure 6 demonstrates the frequency distribution according to severity, of induced mutations in yeast. The estimated ratios of

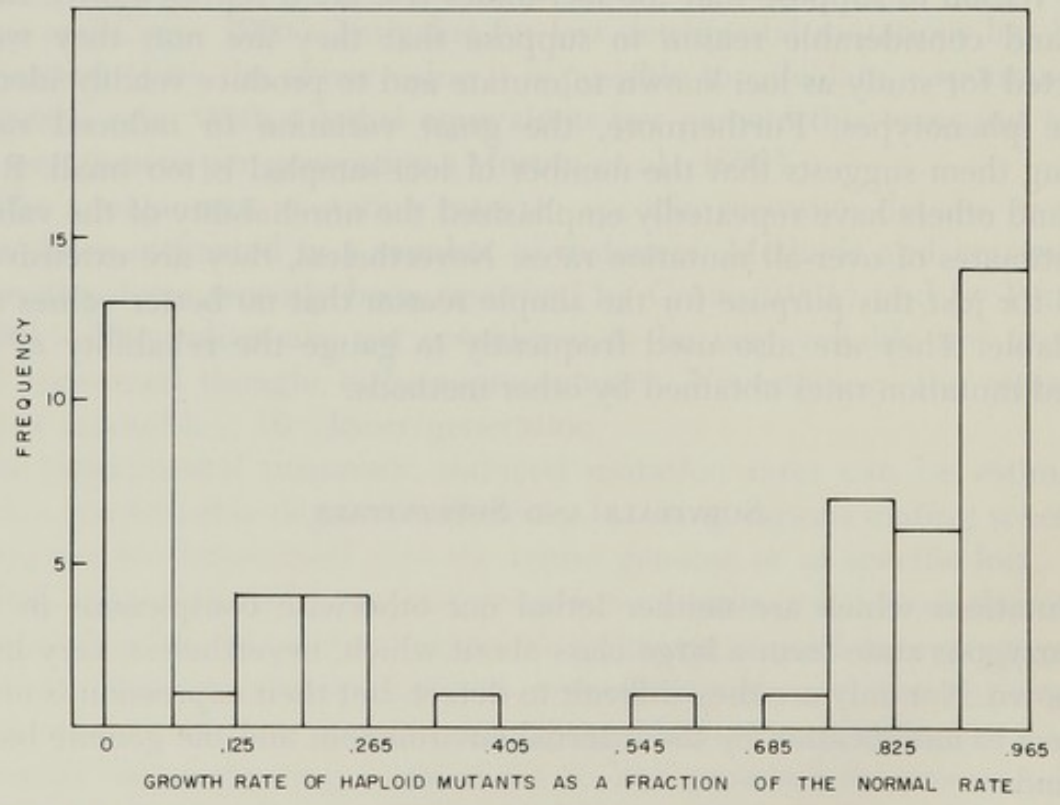


FIG. 6—The distribution, by severity of phenotypic effect, of ultraviolet-induced mutations in yeast (from James, 1959).

detrimentals to lethals has ranged from 3.5 to 0.75. Such values are no doubt gross underestimates; not only do the studies fail to consider all aspects of fitness, but they are unable to detect less severe mutations. Bateman (1959) has estimated the ratio at 66 in an investigation which did not attempt to isolate individual mutants, but rather utilized the increase in the genetic component of the variance. Such studies have provided little evidence of beneficial mutation.

The dominance of less detrimental genes is a matter of some concern. Of the few studies so far made, some have indicated that the dominance of individual mutation is inversely correlated with their severity (Greenberg and Crow, 1960; James, 1960). On the other hand, other investigations have indicated that overdominance, in the form of a selective advantage, is common (Burdick and Mukai, 1958; Mukai, 1961; Wallace and Dobzhansky, 1963).

In estimating polygenic mutation rates, the difficulties introduced by inability to isolate individual small effects is usually overcome by expressing mutation in terms of an increase in the genetic component of the variance (Clayton and Robertson, 1955). The doubling dose is that dose which produces in one generation the same amount of genetic variation as occurs spontaneously. So far, the character most thoroughly studied has been chaeta number in *Drosophila*. Spontaneous and radiation-induced rates have been calculated by various investigators (Durrant and Mather, 1954; Clayton and Robertson, 1955; Paxman, 1957; Scossiroli and Scossiroli, 1959; Yamada, 1961; Yamada and Kitagawa, 1961). Doubling doses have been estimated by Yamada. These vary from 7 to 150r and do not seem to differ from estimates for mutations having conspicuous qualitative effects.

## Radiation Hazards

It is generally agreed that the genetic effects of ionizing radiation, whether of natural or artificial origin, are harmful. The occurrence of beneficial mutation is not disputed, but both logic and experimental results suggest that this is rare. Even if the rate of beneficial mutation were relatively high, the advantageous alleles would be widely distributed because of their selective advantage and any addition to their number would be of little consequence.

Accepting the thesis that an increased mutation rate is to be avoided, it follows that protective measures should be adopted where possible. However, before a rational decision can be made concerning the nature and extent of these measures, it is necessary to answer the question: *How*

harmful are ionizing radiations? There is also the counterpart to this question: *How* difficult or expensive, in human terms, are the measures required to reduce the exposure? Only the first of the two questions will be discussed here. The answer cannot be derived solely from a knowledge of the rate at which mutants are induced by radiation because, once induced, a mutation may be handed on through subsequent generations. This transmission can amplify even a relatively slight effect in any one carrier individual to the point at which the accumulated consequences are sufficient eventually to ensure the elimination of the gene through a death or a failure to reproduce.

Assessments take into account the important aspect of gene transmission by considering mutations, not in terms of their specific effects on phenotype, but rather in terms of their ability to perpetuate themselves in subsequent generations. The procedure permits all mutations to be classified within a single category, the genetic fitness which they confer upon individuals carrying them.

Genetic fitness is obviously related to the degree of detriment imposed by a mutation; if a mutation is harmful it will tend to be eliminated from a population through failure of its carriers to produce a normal number of progeny. Nevertheless, a knowledge of genetic fitness does not always provide a satisfactory measure of the contribution of a mutation to human suffering. Some of the most detrimental mutations, as dominant lethals acting early in embryonic life, may be eliminated unnoticed. Other mutations, as those which produce their effects late in life, may cause much suffering despite a high accompanying genetic fitness.

An intimation of the extent of the hazard can be obtained by a consideration of individual categories of mutation.

Dominant lethals, by definition, are not transmitted through subsequent generations and a direct measure of damage is thus provided by the frequency of their induction. The relative importance of dominant lethals as a hazard of radiation is usually discounted; involving gross chromosome aberration, these mutations tend to occur as a power of the dose at higher doses, and it can be suspected that they are relatively infrequent under conditions of low intensity chronic irradiation. In general, the time at which death occurs in the mouse has been found to be before and shortly after implantation of the embryos (Russell, 1954; Lyon et al., 1962). In man such lethals would probably only cause very early miscarriages. Nevertheless, fetal death due to gross chromosome aberrations has been noted in human subjects (Penrose and Delhanty, 1961; Maclean et al., 1961). Furthermore, the profusion of chromosome aberrations found in test organisms may well mask a frequency of point mutations which would be relatively high by the standards of experiments spe-

cifically designed to detect point mutations. Since these are not passed through a number of generations, they cannot be detected by the classical genetic technique of segregation analysis.

Dominant visible mutations are induced much less frequently than any other class but may be an important hazard of radiation nevertheless. Congenital defects among live and stillborn form a large discrete class, and the effect of radiation on their frequency has been a matter of particular concern. These traits now occur with a frequency in excess of 3 per cent (Neel, 1958; McKeown and Record, 1960). They have sometimes been attributed to dominant mutations because they appear sporadically, yet are not often attributable to homozygosity of recessive alleles. However, there is accumulating evidence that there are many etiological pathways leading to specific defects and that many causal factors are involved (see Fraser, 1961). These include recessive genes of low penetrance, the additive effect of genes at several loci, complex genotypes, unusual maternal environments, and chromosome aberration. Neel (1958) is of the opinion that less than 10 per cent of the defects can be attributed to completely penetrant mutations, whether dominant or recessive. He has considered that many of them are segregants resulting from the existence of heterozygous genetic systems needed to ensure normal development. However, the importance of such systems has been disputed (Morton, 1960).

The fact that surveys have failed to detect induced visible mutations in the progeny of irradiated individuals is not surprising under the circumstances. The results of several surveys for which control populations were available have been published (Macht and Lawrence, 1955; Crow, 1955, Neel and Schull, 1956; Turpin et al., 1956; Tanaka and Okhura, 1958). Such surveys are plagued by uncertainties regarding the gonadal exposure, by the difficulties of obtaining proper controls, and by suspected biases introduced by the methods of collecting data. Among offspring of irradiated parents, an increase in frequency of abnormalities, including abortion and stillbirth, has been fairly consistent but not statistically significant. However, the most comprehensive survey, and that in which the data are least subject to bias (Neel and Schull, 1956), failed to show a significant effect of either paternal or maternal irradiation on malformation or neonatal death.

Assessments of the genetic hazards of recessives, whether sex-linked or autosomal, lethal or nonlethal, is particularly dependent on reliable information about their expression in the heterozygote. Although forming a huge class, these mutations must be too rare individually to exist frequently in the homozygous state, and there can be little doubt that the genetic deaths associated with them are usually a consequence of im-

pairment of the heterozygote. Quantitative data now available on the degree of expression of recessive alleles in the heterozygote have been obtained almost entirely from *Drosophila*. Here dominance is usually estimated from the effect of the allele on some component of fitness, as fertility or ability to reach adulthood. Some induced mutations are actually beneficial in the heterozygote when defined in these terms (Stern et al., 1952; Mukai and Burdick, 1959).

In mammals, direct information on the effects of induced mutations in the heterozygous state can be obtained by comparisons of the progeny of irradiated and unirradiated animals. Results have been disconcertingly variable. A significant decrease in the life span has been noted in the progeny of male mice receiving moderate doses of neutron radiation (Russell, 1957). Spalding and Strang (1962) noted a reduced survival time in the descendants of five generations of irradiated mice, and Cox and Willham (1962) have detected an increase in early death among the progeny of swine following spermatogonial irradiation of 300r. On the other hand, other investigations with mice have failed to detect damage (Lindop and Rotblat, 1962) or have found evidence of what appears to be the opposite (Ramel, 1962).

A similar variability has been found in lower organisms. Some investigators have found significant increases in mean viability, others have found decreases. (Wallace, 1958; Burdick and Mukai, 1958; Mukai, 1961; Falk, 1961). The conflict in these results is probably due in part to experimental and statistical difficulties. However, the effect of external environment and genetic background on the expression of mutant genes have been well demonstrated and it seems likely that these are influential factors (Müller and James, 1961; Kivi and James, 1962; Wallace, 1963).

Probably the greatest difficulties in assessing genetic hazards of radiation are those attending quantitative traits. The effect of an increase in polygenic mutation cannot be estimated until the great amount of genetic variability normally associated with these traits has been explained. It has been calculated that over one third of the variation in birth weight (Robson, 1955) and up to three quarters of the variation in intelligence (Mather, 1956) is genetic in origin. Shifting selection pressures under which different alleles are alternately favored, and a selective advantage of heterozygotes, may be influential. The presence of genes with opposite effects may also be a factor where selection is for an intermediate phenotype. In general, it seems that continuously varying traits are well buffered against the effects of mutation; a permanent doubling of the mutation rate might double the genetic variability but the effect would be small in the first few generations. The impact of such an increase on human subjects can be conjectured only because of ignorance about

which phenotypes are more desirable from either a selective or social point of view. It has been calculated, however, that permanent exposure to a doubling dose would eventually triple the number of children with an I.Q. of less than 70 (Mather, 1956). Direct experimental tests of such a deduction would be difficult in any experimental organism. A reduction in learning ability in rats whose ancestors had received gonadal exposures over a number of generations has, in fact, been observed by McGregor and Newcombe (1962). However, this effect was not necessarily due to induced gene mutations, and further experiments would be needed to rule out possible contributions from gross chromosomal changes and litter-size effects.

## Quantitative Assessments

The mathematical relationship between mutation, genetic fitness and the prevalence of hereditary defects has been presented by Haldane (1937) and Muller (1950). Essentially, the Haldane-Muller principle is an expression of the fact that, under conditions of recurrent mutation, the frequency of mutant individuals within a population tends toward an equilibrium value. When this value is achieved, the entrance of a new mutation is balanced by the loss of an identical mutation through failure of an individual to transmit the damage to his progeny. This loss constitutes a genetic death.

Three general methods are used to obtain quantitative estimates of genetic damage induced by radiation: One utilizes information on the number of loci and their induced mutation rates to estimate the number of "genetic deaths" expected from a given exposure of radiation. A second utilizes the natural incidence of recognized hereditary defects; the hazard is defined in terms of the expected increase in prevalence of these defects. The third method predicts genetic deaths from information on the natural prevalence of recessive damage as determined in consanguinity studies.

The estimates obtained by these procedures have an appearance of reliability which they do not possess; they are not free of subjective reasoning and they should be used only with an understanding of the assumptions and conjectures involved in their calculation. Until the uncertainty factors attached to them can be reduced, they remain too vague to be more than indicative. Meanwhile, they permit the problem of radiation hazards to be regarded with some degree of objectivity and serve a useful purpose in exposing those areas where more intensive research is needed. None of them takes into account the damage resulting from the induction of gross chromosome aberrations.

*Total Genetic Death*

This method is based on the principle that any detrimental mutation, regardless of the degree of detriment that it imposes, will eventually lead to one genetic death. Calculations require a knowledge of the effective number of loci and the mean rate at which mutations are induced at these loci. The prediction covers all damage, and calculations are not hampered by ignorance of past or present changes in selection pressures within a population. It is, however, extremely difficult to relate these genetic deaths to specific phenotypic disabilities within the population. These disabilities can best be considered as resulting in reduced fertility. In the extreme case they lead to death of the organism.

Estimates of the haploid number of loci in man range from 5,000 to 100,000 but center around 10,000. The uncertainty introduced here can be reduced by substituting the ratio of total mutations in the genome of a gamete to the average number at a specific locus. This value has been determined with considerable accuracy in *Drosophila* and is, by coincidence 10,000 (Muller, 1957). As for the induced mutation rate, that derived from mouse studies is considered to be the most applicable. The mean value for seven specific loci is $6 \times 10^{-8}$/gamete/r for low-intensity chronic irradiation of spermatogonia and oocytes. Apart from its suspected unreliability, this value does not include nonvisible detrimentals which may or may not be induced at these loci.

With the above values it can be calculated that the exposure of a population having $10^6$ births per generation to a dose of 5r delivered at low intensity would eventually lead to 6,000 genetic deaths.

*Clinically Identifiable Disabilities*

The second approach utilizes the doubling dose and the prevalence of hereditary defects normally present in a population. The total number of affected persons resulting from application of the doubling dose is equal to the number of affected persons normally present in one generation, provided the population is in genetic equilibrium and provided the defects under consideration are maintained by recurrent mutation. These defects will be spread over many generations, the number depending upon individual genetic fitness.

So far, this approach has received the most attention. It has been adopted in national reports of Great Britain (Medical Research Council, 1956) and the United States (National Academy of Sciences, 1956) and in reports issued by the United Nations (1958, 1962). Its main advantage lies in the fact that predictions are applicable to clinically identifiable

defects; this permits a relatively objective interpretation of the social and personal impact of an increased mutation rate. The estimate does not rely on knowledge of genetic fitness, other than an assurance that a selective advantage is not involved in maintaining the prevalence of a defect. Nor is it necessary to know the number of loci. A disadvantage is the fact that it omits clinically minor effects which are not individually identifiable.

The doubling dose for man can only be conjectured; recent attempts to estimate this value from human data have been more successful in exposing pitfalls than in leading to improved estimates. Data on the shift in sex ratio in the progeny of irradiated mothers has been used to calculate a doubling dose of 30r for sex-linked lethals (Lejeune and Turpin, 1957). However, increased realization of the complexities involved in sex ratio shifts has reduced the reliability placed on such calculations. The absence of a detectable increase in congenital malformations among the progeny of irradiated individuals has been used to provide a lower limit of 10r to the doubling dose (Neel and Schull, 1956). This value is questionable in view of the fact that probably only a small fraction of these defects is attributable to point mutation in the parents. The data thus permit a still lower limit.

The value of 3r for natural background radiation sets a lower limit for chronic exposure. A report of the Medical Research Council (1956) concluded that the doubling dose might be as high as 150r or even higher. The "best" estimate was that the representative value lies between 30 and 80r, and 30r has frequently been chosen as a reasonable value. Some support for this choice is provided by the slope of the dose-effect curve for induction of leukaemias in man (Court Brown and Doll, 1957). Estimates of the doubling dose for point mutations in organisms other than man vary between 8r and 350r but are mostly between 30 and 60r (Newcombe, 1962). For the mouse, acute irradiation of spermatogonia and oocytes yields a mean value of about 30r for seven specific loci. With chronic irradiation, the value is increased to about 85r and is perhaps representative of man.

The most recent survey of the prevalence of genetic disabilities (UN Report, 1962), based on various studies, indicates that about 6 per cent of all live-born suffer at some time during their lives from serious defects in which a major genetic component is known or suspected. About 1/6 of these are known to be caused by gross chromosome aberrations. Something like 1/4 of the remainder are attributable to point mutations which are almost certainly maintained by recurrent mutation. Three quarters of the different single gene traits are due to dominant effects. This high ratio of dominants to recessives contrasts sharply with the relative fre-

quency with which the two types are induced in other organisms and suggests that many mutant recessive genes in human subjects remain to be identified as such. The remainder of the disabilities are congenital malformations or constitutional disorders, the inheritance of which is not well understood. Almost certainly some of these exist as phenocopies, some are a consequence of complex genetic interactions, and many of them probably involve a polygenic component in their heredity. The individual frequencies of certain recessive traits are so high that it is suspected that they have only recently been rendered disadvantageous by environmental changes or that they are not maintained by recurrent mutation. Instances of a selective advantage of detrimental genes are well documented, if rare. The value of 5 per cent thus sets something of an upper limit to the prevalence of recognized defects which would respond to a doubling dose.

Using a value of 85r as the doubling dose and 5 per cent as the prevalence of responsive defects, it can be calculated that a dose of 5r delivered at low intensity to a population which produces $10^6$ progeny in a generation would result in the production of about 3,000 genetically disabled persons.

*Genetic Damage Exposed Through Consanguineous Marriage*

The third approach utilizes the prevalence of genetic ill health as estimated indirectly through information about recessive alleles present in a population. This, as discussed previously, is obtained through surveys of the progeny of consanguineous marriage and can be expressed as lethal or detrimental equivalents. Provided the genes so exposed are maintained by recurrent mutation and their mean genetic fitness is known, it is possible to calculate the amount of genetic death which is expressed each generation in a normal outbreeding population. This is the same as the spontaneous mutation rate (Morton et al., 1956).

The method has an advantage over the preceding one in the efficiency with which it isolates genetically caused defects from nongenetic defects. A major disadvantage is the fact that the defects exposed by inbreeding probably are not typical of the way in which the alleles express themselves in the normal population where most of the genetic death will occur as a consequence of heterozygosis.

Using a value of 0.08 for expressed damage per gamete per generation, a doubling dose of 85r for exposures of low intensity, and a population giving birth to $10^6$ progeny per generation, the estimated number of genetic deaths resulting from 5r is 9,000. The value is an underestimate to the extent that it is based only on alleles causing death between birth

and the age of 30 in the homozygote. It is an overestimate if any of these are not maintained by recurrent mutation, a possibility that has been considered and rejected by Morton (1961).

It is apparent that the close agreement of the three estimates is not an indication that they are reliable. The assessments which consider genetic deaths do not take into account disabilities in the carriers of mutant genes. The number of these carriers goes up as the probability of elimination in any one individual goes down. Thus, with a mean selective disadvantage of these mutant genes of 0.02, there will be 50 times as many carriers as there are eliminations. It is impossible to estimate how many of these would be regarded as seriously affected during their lives. The doubling dose method, on the other hand, attempts to estimate this number. The number of genetic deaths calculated from data obtained from consanguineous studies may, however, be usefully compared with that obtained by the first approach. One would expect the former to be smaller since it is derived only from the frequency of alleles that are effectively recessive lethals. In fact, it is slightly larger.

For a reliable assessment of the genetic hazards of radiation, the areas in need of further investigation are so manifold that improved data in any one of them will scarcely improve the over-all estimate. It is possible, however, to perceive some areas that are in particular need of attention. A better understanding of the influence of spontaneous mutation on the well-being of human populations would provide a firm base for estimating the consequences of exposure to man-made radiation. Such an understanding can be achieved only through investigations of the genetic structure of populations and of the forces which mold this structure. Such investigations are complicated by the fact that human populations are almost certainly not in a state of genetic equilibrium; it must be expected that the great changes in environment to which man has exposed himself in recent times have been followed by a shift in selection pressures. Medical advances and alterations in reproductive patterns are only two of many influential factors. Fortunately, human populations are well suited to studies of the roles of mutation and selection in maintaining the frequency of hereditary disabilities, and efficient techniques for using information contained in the vital and health statistics systems now available and others are being developed (Newcombe et al., 1959; Neel, 1962). Even a reliable quantitative estimate of genetic damage induced by radiation would not necessarily constitute, by itself, a satisfactory measure of the hazard. To be meaningful, the damage must be expressed in terms of its personal and social consequences. Wright (1961) has suggested that the social importance of mutation arising out of the increasing use of potent sources of radiation can perhaps be treated in terms of a

balance between contribution to society and social cost. If man is to receive the maximum benefit from his technological advances, at the minimum cost, it is this balance that must be examined most closely.

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