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Progress in

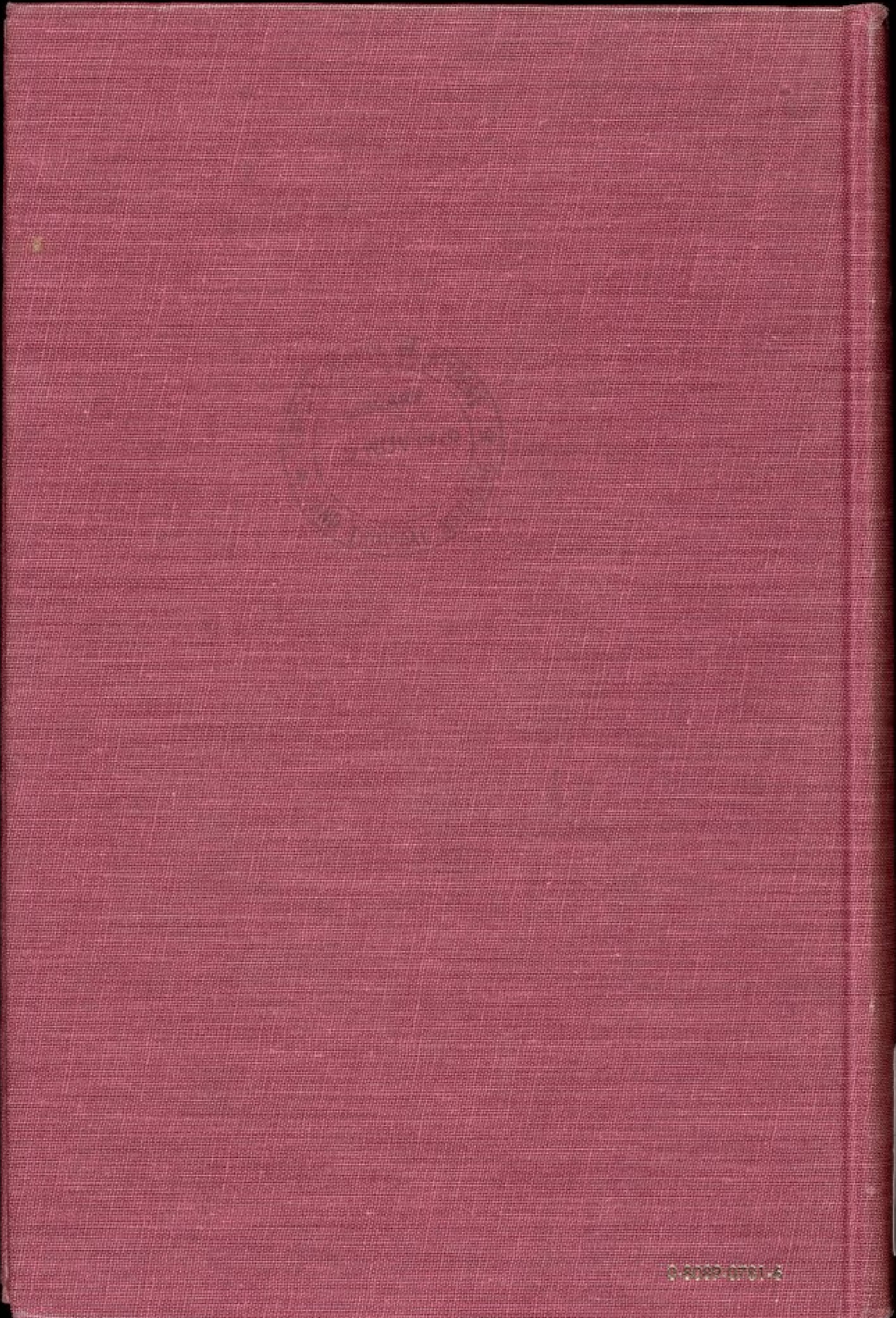
MEDICAL GENETICS

VOLUME IX

Edited by

ARTHUR G. STEINBERG

ALEXANDER G. BEARN



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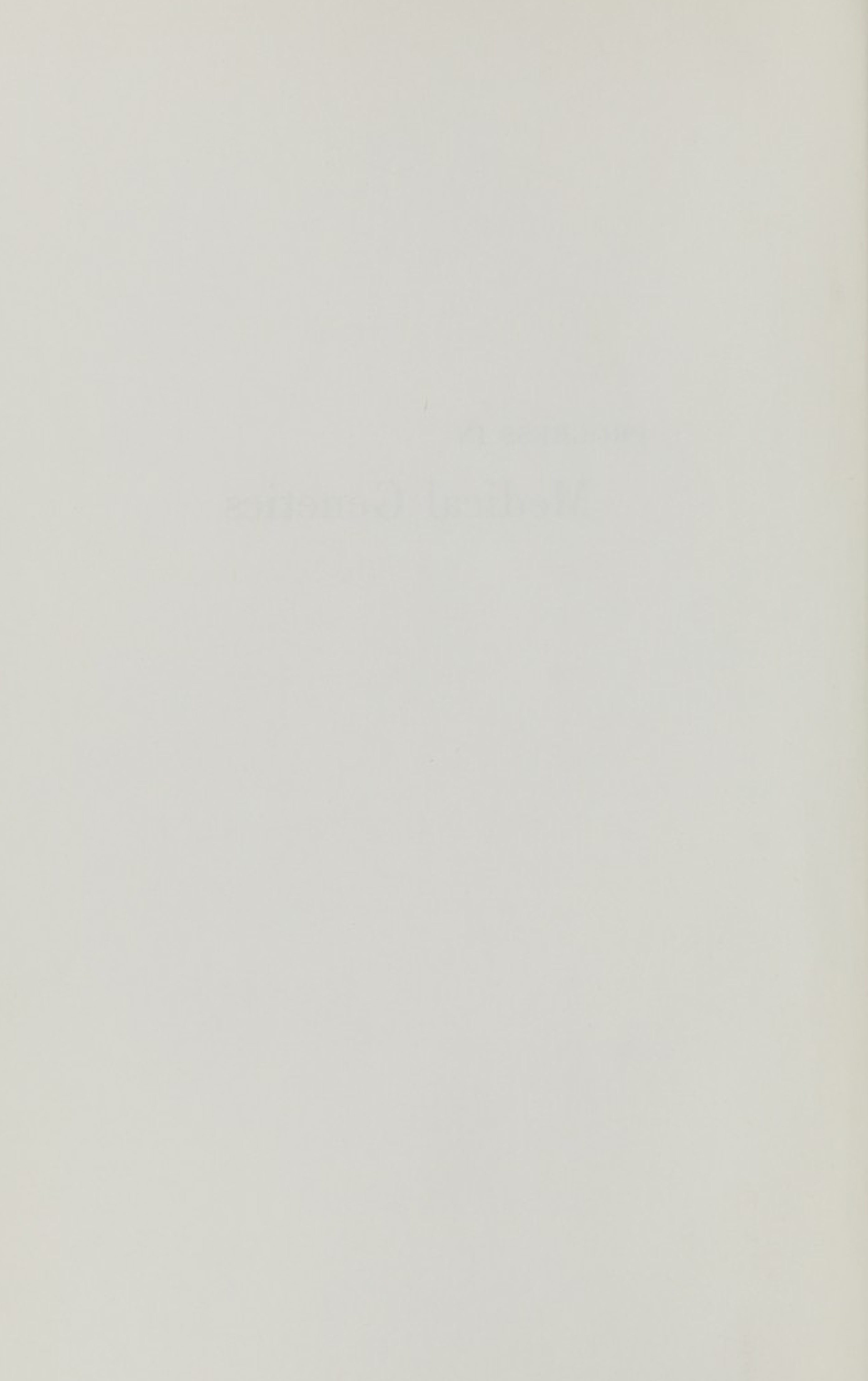

PROGRESS IN

Medical Genetics

Volume IX

Edited by

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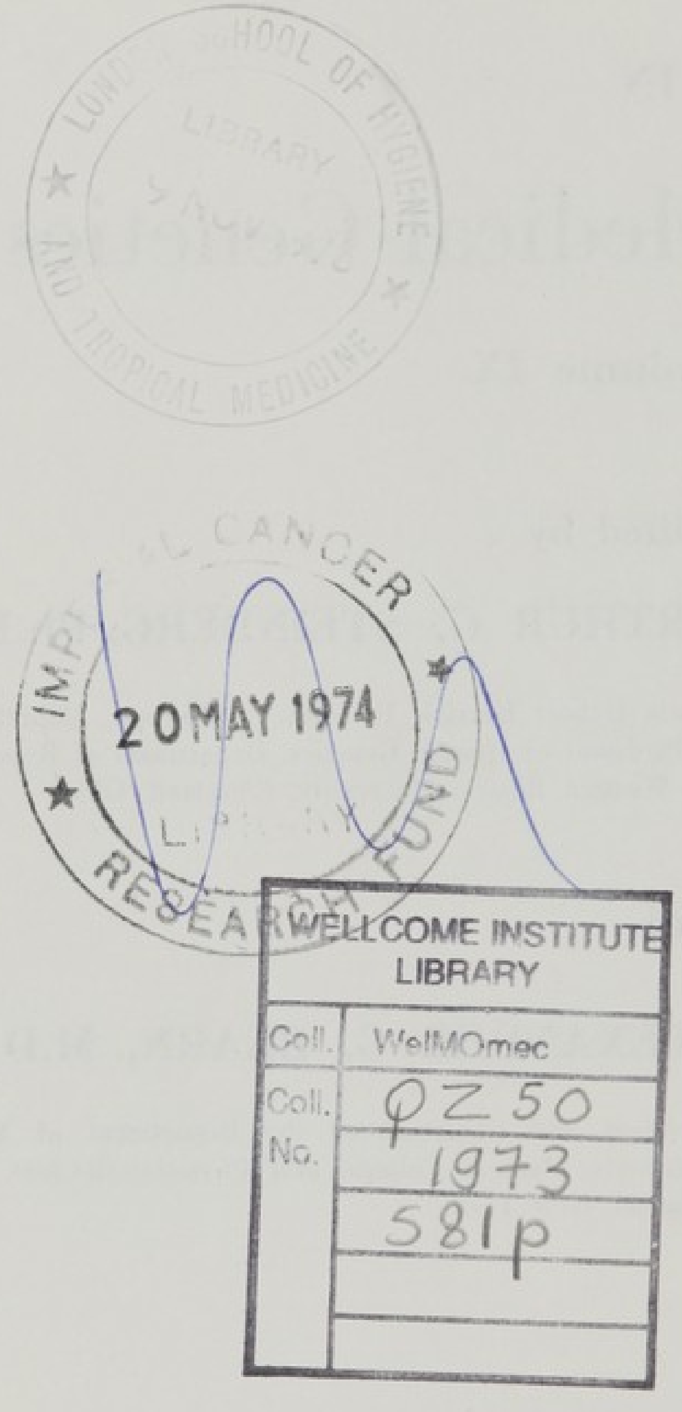

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Foreword

The splendid contributions in this volume clearly demonstrate that the title of this series, *Progress in Medical Genetics,* is more felicitous than was realized when it was selected.

A year or so ago, the drama of human chromosome analysis seemed to have come to an end. Indeed, about three years ago a prominent geneticist said that human chromosome analysis had become a form of routine taxonomy. Now the development of new staining techniques has revitalized the field and opened new vistas for human cytogenetics. Orlando and Dorothy Miller and Dorothy Warburton elegantly describe these exciting advances and weigh their implications (Chapter 1).

Knowledge of the early development of the human and of his genetic control is essential for many aspects of medical care, e. g., understanding of congenital anomalies, fertility control (including enhancement), the causes of twinning, and so on. The development of techniques for in vitro fertilization of the human egg and for maintaining the zygote in vitro for a period of time has provided opportunities for analyses which could not previously be done. These intriguing developments are described by Ruth E. Fowler and R. G. Edwards in Chapter 2.

Alfred G. Knudson, Jr., Louise C. Strong, and David E. Anderson describe, in Chapter 3, the recent development of a new analytical approach to the genetics of cancer in man. Their ingenious approach promises to remove much of the haziness from this very difficult genetic problem. We are pleased to be able to present this clear and exciting review of new developments by the people responsible for them.

The ultimate goal of human genetics is its application in the care of patients. Medical geneticists are becoming increasingly aware that experimentation in methods of application is essential if care of patients with genetic diseases is not to tax the supply of trained personnel (medical and paramedical) and of finances. Outstanding work in this area has been carried on in Montreal for several years. We have been fortunate in being able to persuade pioneers Carol L. Clow, F. Clarke Fraser, Claude Laberge, and Charles R. Scriver to describe their work for our readers (Chapter 4).

Geneticists whose training was completed before 1959 were startled to learn that XXY mammals are male and that XO mammals are female (in *Drosophila* they are female and male, respectively). Since that initial finding, knowledge concerning genetic control of sexual difference in terms of gene action has

progressed at an accelerating rate. Daniel D. Federman, who is responsible for a large portion of these advances, describes them in Chapter 5.

The association of hypercholesterolemia with coronary artery disease and the clear familial correlation of plasma cholesterol levels have stimulated much research on the genetics of this and other plasma lipids and on the diseases associated with their abnormal levels. Robert S. Lees, Dana E. Wilson, Gustav Schonfeld, and Shelley Fleet, workers at Massachusetts Institute of Technology, have been major contributors to our clinical, biochemical, and genetic knowledge of these dyslipoproteinemias. They describe them and evaluate our current knowledge about them with inspiring clarity and insight (Chapter 6).

Earlier in this foreword we remarked on the rapid advances being made in medical genetics. This is dramatically revealed by a comparison of the fine review of pharmacogenetics by Arno G. Motulsky in Volume III with the equally fine and exciting review of the same subject by Elliot S. Vesell in Chapter 7 of this volume.

Once again, we take the opportunity to pay our awe-inspired respects and gratitude to the authors for their splendid articles. We are humbled by their splendor.

We are most pleased to acknowledge the assistance of Dr. Judith R. Tennant as Editorial Consultant.

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

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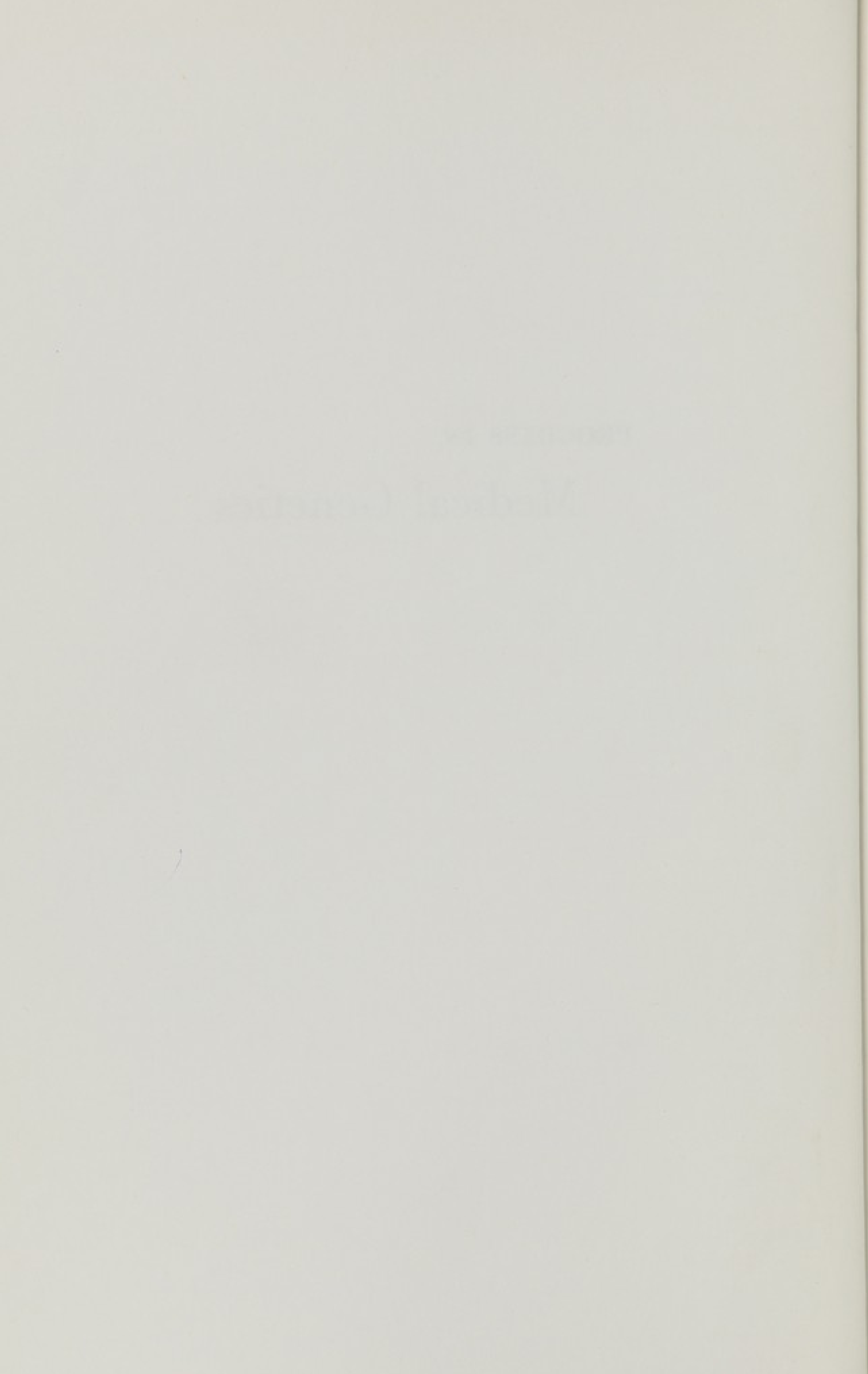

CHAPTER 1

Application of New Staining Techniques to the Study of Human Chromosomes

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This work was supported in part by grants from the National Foundation-March of Dimes, and the U.S. Public Health Service: The National Institute of General Medical Sciences and the National Cancer Institute (GM 18153; CA 12504).

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The past two years have witnessed a revolution in our understanding of the human karyotype. This has been the result of the development of staining techniques which produce chromosome banding so characteristic that every human chromosome can now be identified. The first technique, which was worked out by Caspersson and his associates, involves fluorescence microscopy of quinacrine or quinacrine mustard-stained cells. A flood of new methods has followed, and some of these are so simple and easy to perform that one of them may, in time, replace the fluorescent banding technique. At the present time, however, only the latter method has been proven to give consistent and reliable results, and its applications already cover the entire range of human cytogenetics. The quinacrine fluorescent technique will therefore be described first, with examples of its application in various areas. Other new staining methods will then be described, and their present and potential uses discussed. A continuing development of still newer techniques can confidently be predicted as the implications of the applicability of still other advances in molecular biology become clear.

THE QUINACRINE AND QUINACRINE MUSTARD FLUORESCENCE TECHNIQUES

Mechanism of Action

Binding. Quinacrine (Atebrin) is an acridine dye which was extensively used as an antimalarial agent during World War II, and continues to find limited use as an antiprotozoal agent. Its antimalarial action is based on its capacity to combine with deoxyribonucleic acid (DNA) and block ribonucleic acid (RNA)

synthesis (Kurnick and Radcliffe, 1962; O'Brien et al., 1966). Acridine dyes such as quinacrine (Q) and quinacrine mustard (QM, Fig. 1) combine much more strongly with native DNA than with depolymerized DNA (Morthland et al., 1954). The binding is independent of pH in the range from 3.7-7.4 (Peacocke and Skerrett, 1956).

Acridine and phenanthridium (e.g., ethidium bromide, Fig. 1) dyes combine with DNA by at least two processes (Blake and Peacocke, 1968). The first is by intercalation (Lerman, 1963) of the flat triple-ring systems between adjacent

Quinacrine

Quinacrine mustard

Acriflavine

Propyl quinacrine mustard

Acridine orange

Ethidium bromide

Methylene blue

Azure B

FIG. 1.–Some basic dyes which bind to DNA.

base pairs in the DNA double helix with one of the active basic groups contiguous to a free phosphate radical (Michaelis, 1947). This makes the DNA molecule longer, as demonstrated autoradiographically by Cairns (1962), who showed that T2 phage DNA molecules were as much as 50% longer in the presence of the acridine dye proflavine than in its absence. The maximum amount of acridine dye which can be bound by intercalation, 0.44 molecules per base pair (Peacocke and Skerrett, 1956) (1 quinacrine molecule per 3.8 nucleotides, according to Kurnick and Radcliffe, 1962) is just about the amount expected if intercalation occurs at random and adjacent spaces between base pairs cannot be occupied.

The number of available binding sites for intercalation is lower in deoxyribonucleoprotein (DNP) than in DNA. As protein is removed, especially the fraction which comes away in 0.6 *N* NaCl, primarily histone f1, an increased amount of dye can be bound (Angerer and Moudrianakis, 1972). Although intercalation requires a double helical structure, heat-denatured and cooled DNA binds ethidium bromide and acridine dyes just as well as native DNA, presumably because a sufficient proportion of hydrogen-bonded regions is still present (Waring, 1965). Intercalation is blocked by divalent cations such as Mg^{2+}, which interact with phosphate groups, but is not influenced by base composition, as shown by studies of the interaction of synthetic polynucleotides or various DNAs with quinacrine (O'Brien et al., 1966), acridine orange (Roth and Kochen, 1971) or ethidium bromide (Waring, 1965).

The second method of binding of acridine dyes to DNA involves stacking additional dye molecules on the surface of the double helix by electrostatic interaction with any available negatively charged phosphate groups (Waring, 1965; LePecq and Paoletti, 1967). In the case of quinacrine, this involves the aliphatic diamino side chain, and results in a stabilization of double helical DNA (Kurnick and Radcliffe, 1962; O'Brien et al., 1966).

There is no evidence that cationic dyes, and particularly quinacrine, bind significantly to components of chromosomes other than DNA. The fluorescence of quinacrine mustard-stained fixed chromosomes is abolished by DNase but not by removal of RNA with RNase or removal of histone proteins with 0.2 *N* HCl for 1 hour (Comings, 1971). Proteolytic enzyme treatment (pronase for 1 hour at 37°) modifies the apparent binding of quinacrine by producing even fluorescence, but the basis of this effect is unclear (Comings, 1971). Milder proteolytic treatment with trypsin, sufficient to produce banding with Giemsa stain (see p. 25) has no effect on quinacrine staining (Warburton, unpublished data). Much lower concentrations of the difunctional alkylating agent QM or the monofunctional propyl QM (Fig. 1) are needed to produce intense fluorescence of chromosomes than Q alone. This may be due to specific binding of the alkylating derivatives to the N-7 position of guanine (G), which is by far the most reactive site in purified DNA, although the N-3 and N-7 positions of

adenine (A) are also reactive (Lawley, 1966). Inadequate information is available on alkylating reactions with nucleoproteins (Shapiro, 1969) to speak with assurance of the binding of QM to chromosomes. It is clear, however, that in mammalian chromosomes the fluorescent banding patterns produced with QM are virtually identical to those produced with quinacrine (Miller et al., 1971a; Pearson et al., 1971; Sumner et al., 1971a), in spite of the earlier experience of Caspersson et al. in *Trillium* (1969a).

Fluorescence. Most acridine and phenanthridium dyes are fluorescent and retain this property when bound to DNA. Quinacrine fluorescence, and presumably that of similar dyes, is dependent on the double-stranded nature of DNA (Lerman, 1963; Weisblum and de Haseth, 1972). The interaction of the dyes with DNA, or synthetic polynucleotides (Steiner and Beers, 1959), can produce significant alterations in fluorescence. The fluorescence of ethidium bromide is markedly enhanced by binding to DNA (LePecq and Paoletti, 1967). This enhancement involves only the dye that is bound by intercalation (Angerer and Moudrianakis, 1972). The opposite effect, quenching of fluorescence, is sometimes observed and appears to be related to base composition. For example, at high ionic strength, which permits dye binding only by intercalation, quenching of acridine orange fluorescence occurs with the synthetic nucleotide polyadenylic acid (polyA) but not with polycytidylic acid (polyC) (Steiner and Beers, 1961). DNA rich in adenine and thymine (A+T) has been reported to enhance acriflavine, proflavine, or quinacrine fluorescence, whereas DNA rich in guanine and cytosine (G+C) quenches the fluorescence (Tubbs et al., 1964; Thomes et al., 1969; Weisblum and de Haseth, 1972). The intensely fluorescent quinacrine-stained region of the Y chromosome in an insect of the family Drosophiladae, *Samoaia leonensis,* appears to be composed almost solely of A and T bases (Ellison and Barr, 1972).

Many of the usual fixatives, including 95% ethanol, osmic acid, and 10% Formalin, are said to produce quenching of fluorescence, and freeze-drying may thus provide brighter fluorescence (Bruyn et al., 1950). Acridine dye fluorescence is quenched in basic solution; so these dyes should be used at an acid pH (Metcalf and Patton, 1944; Steiner and Beers, 1959).

Method

Either quinacrine or one of its alkylating derivatives, such as quinacrine mustard, can be used to produce chromosome banding. Metaphase preparations fixed in 3:1 methanol:acetic acid are stained in a 0.5% solution of quinacrine or a 0.005% solution of QM for 5-10 min, washed in tap water, and wet mounted. Good results have been obtained with a variety of buffers or even water as a mounting medium. Fluorescence microscopy is carried out using an HBO 200 W high-pressure mercury lamp with a BG-12 excitor filter and a 530 nm barrier

filter. Because of fading of the fluorescence, as well as to facilitate comparison among chromosomes and cells, it is important to have a photographic record of each cell. The requirements for the most suitable photography are clearly described by Breg (1972).

Chromosomes can be recognized by the consistent pattern of brightly and dully fluorescing bands, which are identical in homologs. For best visualization of the bands, the chromosomes should be extended with chromatids lying closely together. In more contracted chromosomes several bright bands may be seen as one single bright area, although the over-all pattern may still allow chromosome identification.

The Human Karyotype

Normal Chromosomes. Caspersson and his associates (1970g) arranged the fluorescent human chromosomes into a karyotype in which the number assigned to each chromosome corresponded, where possible, to the number which had been assigned previously by other methods of identification. This karyotype has been accepted as the international standard (Paris Conference, 1971) (Fig. 2). The chromosome pairs were arranged first by morphological characteristics (primarily length) and centromeric index. Chromosomes 4, 5, 13, 14, 15, 17, 18,

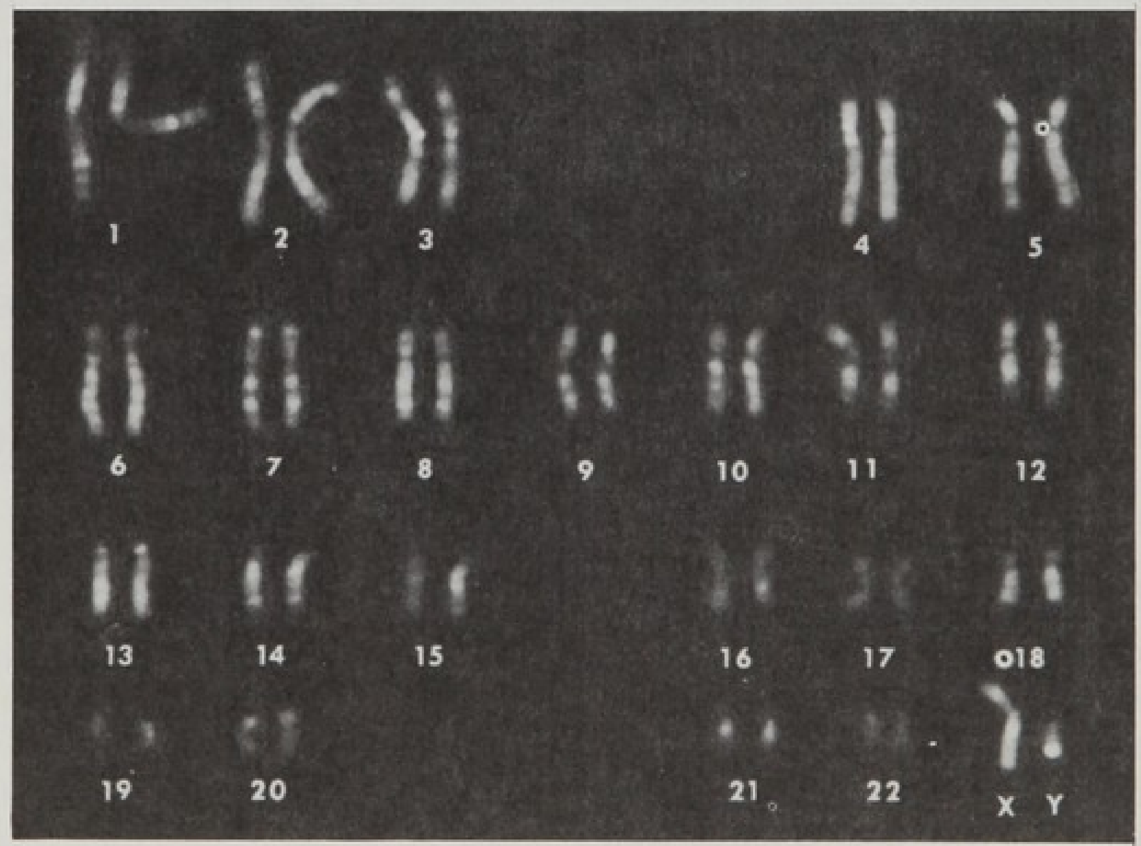

FIG. 2.–Karyotype of a metaphase cell from a male, stained with quinacrine. Note the characteristic banding patterns of each chromosome pair. From Breg, 1972. (By permission of Williams & Wilkins Co.)

and the X were given numbers which corresponded to those assigned on the basis of terminal DNA replication studies. Chromosomes 1, 9, and 16 were oriented so that the secondary constriction was in the long arm. (Some early karyotypes show chromosomes 1 with the long arm at the top.) Since insufficient criteria existed for distinguishing between chromosomes 10 and 12 or between 19 and 20, these numbers were assigned arbitrarily. For historical reasons the chromosome which is trisomic in Down's syndrome (trisomy 21 syndrome) continues to be called number 21, although number 22 is slightly longer (Hamerton, 1971). A brief description of the banding pattern of each chromosome pair has been presented by Caspersson et al. (1971g).

In addition to visual methods, Caspersson and his associates (1971f) have utilized photometric methods involving the production of densitometric tracings from photographic negatives or prints for analyzing the intensity of fluorescence along chromosomes (Fig. 3) and have used Fourier analysis by computer as a means of synthesizing the resultant patterns. In this way, the pattern type of each C-group chromosome has been delineated and differentiated from that of every other C-group chromosome. Thus, accurate automatic machine recognition and classification of human chromosomes is now possible and can be expected to become available to many workers before very long. Television techniques can also be used, instead of the photographic method of recording fluorescence patterns, with resultant speed-up of large-scale classification work (Caspersson et al., 1970c). In the meantime, visual analysis of fluorescent banding patterns provides a very adequate means of chromosome identification.

Common Variants. Some of the chromosomes have been found to have variant forms in the normal population. These variants are usually marked by a different size or intensity of fluorescence of a specific band. It is assumed that they are inherited in simple Mendelian fashion and could be used in genetic marker studies. For example, Caspersson et al. (1970h) reported that a mother and son each had a D-group chromosome with very bright fluorescence in the short-arm region. Analysis of the variants is complicated by the fact that the degree of brightness or the size of the very bright region appears to be a continuous variable in the population, though not in the individual.

An intensely bright fluorescent band may be present in the pericentric region of number 3 (Fig. 2). Schnedl (1971b) reported finding such a bright region of the number 3 chromosomes in 59% of 50 individuals, including those who had two, one, or no bright band. The long arm of chromosome 4 sometimes has a bright band adjacent to the centromere. This can be an aid in distinguishing chromosome 4 from 5. Each of the acrocentric chromosomes may have bright satellites or short arms. The presence of a bright centromeric region on number 13 is a particularly common variant (Fig. 2). The variant forms of the other D- and G-group chromosomes, e. g., chromosome 14 in Fig. 11, are less common and may be more useful as somatic markers. Examples of each of these may be found in Evans et al. (1971).

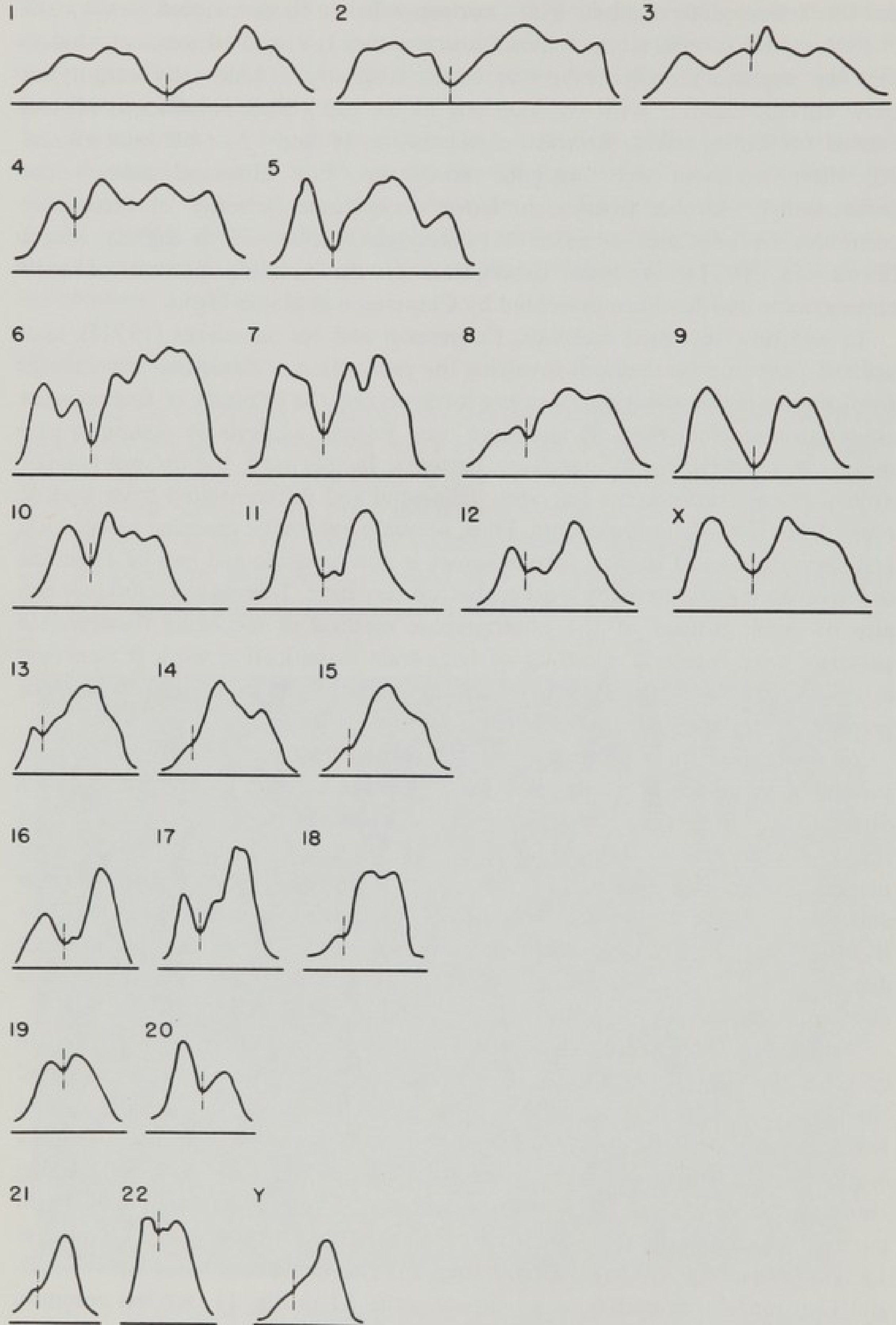

FIG. 3.–Photometrically determined quinacrine mustard fluorescence patterns for the 24 types of human metaphase chromosomes. (From Caspersson et al., 1971g.)

The variant forms of the Y chromosome have received considerable attention. Caspersson et al. (1970h) reported that the length of the intensely fluorescent material of the Y chromosome in a given male was constant. There have been reports of very small Y chromosomes which had little or no intensely fluorescent region (Borgaonkar and Hollander, 1971; Lewin and Conen, 1971; Robinson and Buckton, 1971; Wahlström, 1971). Very long Y's which had a long region of intense fluorescence have also been reported (Knuutila and Gripenberg, 1972; Robinson and Buckton, 1971; Wahlström, 1971; Wilson et al., 1971). Bobrow et al. (1971) and Schnedl (1971c) measured the dully and intensely fluorescent segments of Y chromosomes from normal males. The length of the very bright, but not of the dull, region varied widely; this variation accounted for almost the entire difference in total length of the Y chromosomes.

Additional intensely fluorescent material in the Y chromosome may be present as several bands. Kim et al. (1971) noted the presence of two brightly fluorescent bands in the long arm of the Y chromosome in 5 of 21 men studied. Walhström (1971) reported finding a father and son both of whom had very long Y chromosomes with four intensely fluorescent bands.

Chromosomal Abnormalities

Autosomal Abnormalities. i. Trisomy. The identity of an additional chromosome can be determined easily by the quinacrine fluorescence method because the entire banding pattern of the chromosome can be observed. Down's syndrome has been extensively studied (Caspersson et al., 1970b; Alfi et al., 1971; Breg et al., 1971b; Mikkelsen, 1971; O'Riordan et al., 1971; Schwinger et al., 1971b). In each case the extra G-group chromosome was a number 21, the shorter chromosome with a bright band in the long arm. Down's syndrome (trisomy 21 syndrome), therefore, invariably involves chromosome 21 and never chromosome 22. In some cases a variant number 21 with a bright satellite has been present in duplicate. The presence of such markers made it possible to determine that the additional chromosome was of maternal origin in one case (Licznerski, and Lindsten, 1972).

A few other types of autosomal trisomy have been investigated. In a case with C trisomy/normal mosaicism, the extra chromosome was identified as a number 8 by its fluorescence pattern (Grouchy et al., 1971). Caspersson et al. (1972b) studied 4 cases of trisomy of this same chromosome.

The D-group chromosomes in 2 patients with the 13-trisomy syndrome included three chromosomes with the fluorescent banding pattern expected for number 13. These chromosomes also had the autoradiographic terminal labeling pattern of chromosome 13 (Miller et al., 1971a). Two apparent D trisomies were shown by fluoresence analysis to have partial (tertiary) trisomy of the long arm of chromosome 14 as a result of nondisjunction in a parent heterozygous for a reciprocal translocation (Allderdice et al., 1971; Miller et al., 1971a; Breg et al., 1972a).

ii. Centric fusion translocations. Identification of chromosome segments translocated to other chromosomes has been possible because the translocated segments retain the banding pattern characteristic of the same segments in the chromosome of origin. Translocations which involve entire chromosome arms are relatively easy to identify, and the translocations which have been studied most extensively by the fluorescence technique are those of the centric fusion types: D/D D/G, and G/G. A wide variety of combinations has been demonstrated: 13/13 (Miller et al., 1971a), 13/14 (Caspersson et al., 1971d; Miller et al., 1971a), 14/14 (Caspersson et al., 1971d), 13/21 (Caspersson et al., 1971d; O'Riordan et al., 1971), 14/21 (Alfi et al, 1971; Caspersson et al., 1971d; Miller et al., 1971a; O'Riordan et al., 1971), 15/21 (Alfi et al., 1971), 21/21 (Alfi et al., 1971; Breg et al., 1971b; Caspersson et al., 1971d; O'Riordan et al., 1971), and 21/22 (Caspersson et al., 1971d; Mikkelsen, 1971). In two translocations, previous identification by autoradiography was found to be in error. A 14/21 had been misidentified as a 15/21 (Miller et al., 1971a) and a 13/21 misidentified as a 14/21 (Caspersson et al., 1971d).

iii. Reciprocal translocations. Translocations which involve smaller segments of chromosomes are difficult to analyze unless both translocation chromosomes are available for study. If both products are present very small exchanges can be identified, such as the addition of the final band of the tip of the long arm of chromosome 1 to the short arm of chromosome 18 (Breg et al., 1972b). Reciprocal translocations rarely involve whole arms of biarmed chromosomes, although this appeared to be the case in 2 patients studied by Breg et al. (1972b). In one the short arms of chromosomes 2 and 6, and in the other short arms of chromosomes 11 and 17, were exchanged, as shown by fluorescence studies. Breg et al. (1972b) have pointed out that even some apparently cytologically balanced translocations may produce an abnormal phenotype, perhaps as a result of a position effect, although a small deletion cannot be ruled out. Francke (1972) has studied a series of translocations, and has discussed whether these translocations are indeed reciprocal. However, it appears unlikely that the present techniques can be used to settle this question, because of their limited resolution.

Breg et al. (1972a) have reported on abnormalities of individual chromosomes in the C group; this is of particular interest since these chromosomes were previously identified only by group. Abnormalities were found for each chromosome in the group with the exception of number 12. One of these translocations involved three chromosomes: 6, 14, and 20 (Allderdice et al., 1971) (Fig. 4). A 6q− chromosome from which half of the long arm had been deleted and a 14/6 translocation chromosome were undetected in regular Giemsa preparations of this woman. An extra D-group chromosome in her child was identified as a number 14 by autoradiography; the extra chromosome, which was a 14/6, did not appear abnormal by this method because the replication

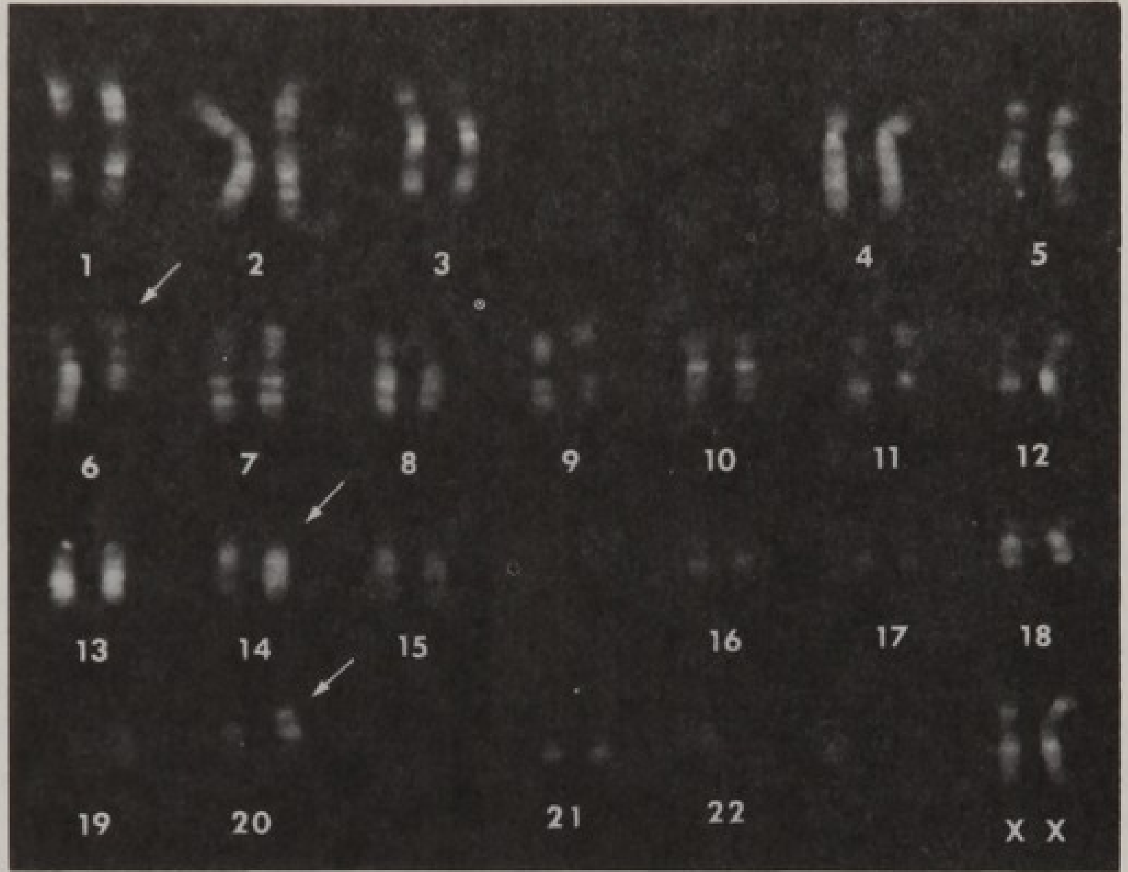

FIG. 4.–Quinacrine fluorescent karyotype of a female with three abnormal chromosomes, indicated by the arrows. The distal half of the long arm of number 14 has been translocated to the short arm of number 20, and the distal half of the long arm of number 6 has been translocated to the centromeric portion of number 14. (From Allderdice et al., 1971. By permission of Springer-Verlag, New York.)

pattern of the translocated portion of number 6 was similar to that of the 14 material it replaced.

In some cases additional methods of analysis have been used in conjunction with quinacrine fluorescence. Bobrow and Pearson (1971) showed the suspected breakpoint in a 4/18 translocation by means of densitometer tracings. The photograph of the translocation chromosome was omitted from this report, and it is unclear whether the breakpoint could be located more precisely by these tracings than by visual analysis.

Malpuech et al. (1971) reported a case in which the fluorescent pattern of a 21/18 translocation resembled that of chromosome 9. In this case Giemsa preparations proved useful: the achromatic area in the short arm of the 21 was visible in the translocation chromosome indicating that the centromere of the abnormal chromosome was derived from the 21 rather than the 18. Evans et al. (1971) illustrated the chromosomes in a t(22p+;9q–) in which the breakpoint in each chromosome was near or within a secondary constriction.

iv. Deletions. A few reports have appeared dealing with recognition of

deleted chromosomes. Caspersson et al. (1970e) reexamined a patient with 5p– syndrome and showed that the banding pattern of the deleted chromosome was that of number 5. They pointed out that the break in the short arm was in the region of a highly fluorescent band. The significance of this finding is not clear since Miller et al. (1969) showed that the breakpoint in the short arm of chromosome 5, as well as in number 4, varied widely among patients with partial deletions of the short arm of these chromosomes.

Deletion of an interstitial segment of a chromosome 13 was demonstrated by the fluorescence pattern in a 13q– case reported by Miller et al. (1971a). The distal bright bands of chromosome 13 were present but the dully fluorescent proximal region was absent. This was in agreement with the finding that the chromosome was late replicating; since only the distal half of the chromosome is late replicating, an interstitial segment must have been deleted. Faed et al. (1972) correlated the absence of part of the long arm of chromosome 18 with absence of IgA and hypothyroidism.

Caspersson et al. (1970a) examined 7 cases in which a Philadelphia chromosome was present; in each case the fluorescent pattern showed that this deleted chromosome was derived from number 22. Similar results were found in 4 cases studied by O'Riordan et al., (1971).

v. Other abnormalities. The fluorescent banding pattern of ring chromosomes is often unclear, and identification of a ring chromosome is usually made by finding an unpaired homolog. However, in the case of a ring 7, identification was made directly because the distinctive bright bands of chromosome 7 were still present. It appeared that only a small segment from the ends of the arms could have been deleted in forming the ring (Breg et al., 1972a).

Fluorescent studies showing an inversion of a chromosome segment have been reported for number 8 (Breg et al., 1972a). The pattern was not distinct, and identification was made by finding a single normal number 8. An inversion in a chromosome 3, however, could be directly identified since some of the intense fluorescent material usually located in the pericentric region was displaced to the middle of one arm (Evans et al., 1971).

The origin of very small abnormal chromosomes is difficult to determine from the fluorescent pattern. There have been several reports of chromosomes in the F- or G-group size range which could not be identified by their fluorescent pattern (Caspersson et al., 1970b, 1971d; Breg et al., 1971b).

Sex Chromosome Abnormalities. *i. The X chromosome.* The X chromosome has a single fluorescent band in the midportion of the short arm and a bright band symmetrically placed in the long arm. It is therefore difficult to distinguish between the formation of an isochromosome for the short arm of an X and a deletion of part of the long arm. Similarly, the banding pattern of an isochromosome for the long arm of an X might appear similar to that of an addition to the short arm. The Xqi and Xpi chromosomes studied by Caspersson

et al. (1970d) showed fluorescent patterns consistent with their designation as isochromosomes, but the fluorescent patterns could not be used to exclude alternative explanations. A ring X chromosome was reported in the same publication. Buckton and her associates (1971) described an X/14 translocation in which part of the short arm of the X was attached to the distal end of chromosome 14. Allderdice (personal communication) established that an X/autosome translocation had the banding pattern expected if the long arm of the X had been translocated to the distal end of the long arm of chromosome 14, with minimal reciprocal exchange. In both cases, the normal X was inactive in females heterozygous for this translocation, whereas a translocation chromosome was inactive in individuals with an unbalanced karyotype.

ii. The Y chromosome. The fluorescent technique has proved particularly suitable for study of the Y chromosome because the distal half of the Y is intensely fluorescent. Osztovics et al. (1971) and Neu et al. (1971) used fluorescence to identify the seven small acrocentrics in patients who proved to be 48,XYY,21+. In 3 cases in which dicentric Y chromosomes were joined by the short arms, both the distal ends showed intense fluorescence (Borgaonkar and Hollander, 1971; Bühler et al., 1971a; Robinson and Buckton, 1971). In another case studied by Bühler et al. (1971b), a girl with only one normal X was found to have a small metacentric chromosome with intense fluorescence over only one end, an area which was also late replicating. This has been interpreted as a Y/autosome translocation. Caspersson and his associates (1971c) studied a definite Y/autosome translocation. Its presence was suspected when fluorescence analysis showed no intensely stained area in the Y chromosome in 4 of 7 cases with XO/XY mosaicism. In 3 of the 4 cases the father had a normal-appearing Y with the usual intense fluorescence of its distal segment. A simple deletion seemed unlikely, because each of the derivative Y's was longer than the nonfluorescent segment of the paternal Y. Meiotic studies in the one sexually normal 45,X/46,XY male showed the presence of a quadrivalent (with the long arm of the Y chromosome pairing with the second largest bivalent and the short arm of the Y pairing with the X). Further analysis of this figure, with the aid of a centromeric heterochromatin staining method to be described later, showed that a Yq/2p translocation was present. By implication, a Y/autosome or X/Y translocation may be present in each of the other patients, or in any individual who has a Y chromosome which is structurally different from the father's. Ferguson-Smith (1966) suggested that an X/Y translocation may be present in sex-reversed XX males. A large number of these individuals have been studied by quinacrine fluorescence, but in none has any intensely fluorescent material been seen on either X (Caspersson et al., 1971a; George and Polani, 1970) or elsewhere in the karyotype (Andersson et al., 1972; Breg et al., 1971a). Zuffardi et al. (1971) noted that the normally brightly fluorescent segment of the short arm of the Y in *Drosophila melanogaster* is no longer bright when this segment is

translocated to the X or autosome III, and they urge caution in interpreting the karyotype on the basis of the intensity of a fluorescent band.

Morrillo-Cucci et al. (1971) described a long Y chromosome in which the distal tip was dully rather than brightly fluorescent. The patient was a 45,X/46,XYq+ mosaic with gonadal dysgenesis, whose father had a normal Y chromosome. We think this chromosome, with its dull distal tip, is more likely to be the result of a Y/autosome translocation than a duplication of the long arm of the Y, as suggested by the authors. Fraccaro et al. (1971) reported a case with a small metacentric chromosome, one end of which was intensely fluorescent, which was apparently the result of an inversion of the Y chromosome. Robinson and Buckton (1971) presented a series of well-studied cases in which quinacrine fluorescence analysis permitted unquivocal demonstration of specific structural abnormalities of the Y. These included duplication of intensely fluorescent long-arm material, short and long dicentrics, and an inversion. Two patients had isochromosomes for the long arm of the Y and a female phenotype. A third, a subfertile male, had a tiny ring Y. The phenotypic correlations in cases like these support the idea that the male determining factors carried by the Y are on its short arm.

Y bodies in Interphase Nuclei

The fluorescent staining technique can be used to identify the Y chromosome in interphase nuclei. Caspersson et al. (1970h) and Pearson et al. (1970) demonstrated an intense fluorescent spot present at the periphery of interphase nuclei in males but not in females. This has been referred to as the Y body or Y chromatin. Several quantitative studies have been reported. Schwinger et al. (1971a) studied hair root sheaths from 52 normal males and found 15-64% of the cells positive for Y bodies; 60 of the 5200 cells showed two Y bodies, and they were "rare" in cells from females. Cervenka et al. (1971) examined hair sheaths in 10 cases and found 78-90% positive cells in 8 males and none in 2 females. Robinson (1971) examined 463 consecutive newborns. The 239 males averaged 60-70% positive cells with about 10% duplexes (less than ¼ the diameter of the nucleus apart) and a very small number of double or multiple spots; the 224 females had an average of about 5% positive cells. Greensher et al. (1971) developed a method using Wharton's jelly to test for both X and Y chromatin bodies. The Y bodies were very intensely stained and were 0.25-1.0 microns in size, whereas the X bodies were less intensely stained and were 1.0-3.0 microns in size. The Y bodies were present in 75% of the cells in males but absent in females; X chromatin was found in 25-50% of the cells in females. The results, checked by restaining with carbolfuchsin, indicated that there were no errors in sexing 1761 consecutive neonates by the fluorescence technique.

Barlow and Vosa (1970) studied air-dried smears of spermatozoa that had

been treated for 5 min in absolute ethanol and stained for 20 min in quinacrine or quinacrine mustard. The basal third of the sperm head consistently had brighter fluorescence, and this was separated by a sharp boundary. In about 40% of the quinacrine-stained sperm heads and 46% of the quinacrine mustard-stained sperm heads there was, in addition to a still brighter fluorescent body, the F body. Because of the regular position of the F body, at the boundary of the fluorescent part of the sperm head, and its frequency, which resembles the expected 50% of Y-bearing sperm, the authors believe this to be the chromatin of the distal segment of the long arm of the Y. That is, their F body corresponds to the Y body. Confirmatory evidence was presented by Sumner et al. (1971b), based on the lower DNA content of sperm containing a Y body as compared to that of sperm presumably containing an X chromosome. They also noted that more than 1% of sperm in samples from all 9 individuals tested had two brightly fluorescent bodies. Their interpretation of this as indicating the presence of two Y chromosomes is supported by studies of XYY males.

Somatic cells from males with two Y chromosomes may have two Y bodies (Pearson et al., 1970; Robinson and Buckton, 1971; Rainer et al., 1972) (Fig. 5). Hultén and Pearson (1971) examined primary spermatocytes from an XYY male and found that 45% of the cells had two Y bodies. There was also an increased frequency of YY sperm (having two Y bodies). Diasio and Glass (1970) reported that in sperm from an XYY male, 70% had a single Y body and 5% had two Y bodies.

Several studies have been carried out to test the accuracy of using Y bodies present in amniotic fluid cells to predict the sex of the fetus. Cervenka et al. (1971) examined 11 amniotic fluid samples (eight at delivery) and found that in 8 males 41-87% of the cells were positive for a Y body, whereas cells from the 3 females had no Y bodies. Dörner et al. (1971) reported that in 37 amniotic fluid samples from males 5-82% of the cells had Y bodies, and that in the samples from 23 females, no cells had Y bodies. Walker et al. (1971) examined 50 amniotic fluid specimens from fetuses aged 14 weeks to term; the 31 males had 40-84% of cells positive for Y bodies, whereas the females had 0-14%. One fetus in which 74% of the cells were positive for the Y body, and in which some cells were also X-chromatin positive, was shown at birth to be XXY.

Mukherjee et al. (1971) grew amniotic fluid cells on slides and found that 75-84% of the cells from males had Y bodies (including 4-31% with two) whereas 44-74% of the cells from females had fluorescent X bodies.

Attempts to demonstrate the sex of the fetus by looking for Y bodies in the cervical mucus (Shettles, 1971) or the peripheral blood of the mother (Zimmerman and Schmickel, 1971) have been unsuccessful since the observed frequencies are in the same range as that normally found in females, indicating that maternal cells are being scored (Bobrow and Lewis, 1971).

The presence of a Y chromosome can lead to the appearance of a small,

FIG. 5.–Quinacrine-stained buccal mucosal cell from an XYY male, one of monozygotic twins described by Rainer et al., 1972. (Picture courtesy of L. F. Jarvik, New York State Psychiatric Institute and Columbia University.)

brightly fluorescent projection in polymorphonuclear leukocytes, similar to drumsticks in females. Lamborot-Manzur et al. (1971) reported that an average of 19% of the cells in 33 normal males had such projections. Two projections were present in 5-9% of the cells in 3 XYY males, and large projections were found in one male with a large Y.

Meiotic Chromosomes

Fluorescence analysis appears to have a limited role in meiotic studies. Caspersson and his associates (1971e) tried to identify the chromosome bivalents in male meiotic figures by their fluorescent banding patterns. They prepared 50 karyotypes from 500 countable primary spermatocytes in biopsy specimens from 3 males. Although all bivalents could be identified, less than half could be consistently identified. These were 1, 2, 3, 9, 19, 20, 21, 22, and the XY bivalent. The remainder could usually be identified only as to group, i.e., B, C,

D, or E. This method was used by Caspersson et al. (1971c) to show the presence of a Y/autosome translocation.

Pearson and Bobrow (1970a) used the fluorescent technique to show that the short arm of the Y associates with the X during meiosis. They examined 50 cells in diakinesis. In those where X and Y chromosomes were associated, the highly fluorescent region was at the end of the sex bivalent and not in an interstitial position. When X and Y were not associated, part of the Y was intensely fluorescent, as it is in mitotic cells, thus justifying the conclusion that the fluorescence of the Y chromosome is the same during meiosis as in mitosis, and that the long arm of the Y is at one end of the sex bivalent. Pearson and Bobrow (1970b) observed that the distal end of the long arm of the Y is highly fluorescent in meiotic preparations through all stages from spermatogonia to mature spermatozoa.

Tumors and Cell Lines

The fluorescent banding pattern of each normal chromosome is the same in all the cell types which have been studied (Caspersson et al., 1972a). Manolov et al. (1971a) found no difference in pattern between the chromosomes from cultured normal lymphocytes and the normal chromosomes from biopsies or cultures from patients with Burkitt's lymphoma. Manolov et al. (1971b) identified, by their quinacrine fluorescent patterns, marker chromosomes derived from chromosomes 1 and 3, but suggested that a chromosome 14 with an extra distal band is more consistently present in the Burkitt tumors (Manolov and Manolova, 1972). Fleischmann et al. (1972) reported multiple chromosome changes in a lymphosarcoma.

In the work reported by Steel (1971) and by Miller et al. (1971d) some cell lines were shown to have normal diploid complements (e.g., WI-38) and others had a variety of chromosomal abnormalities. The lymphoblastoid line, WI-L2, had a single change: substitution of a biarmed chromosome for a number 21 (Miller et al., 1971d). The Burkitt lymphoma line RAJI had three number 7 chromosomes but a single number 8, a change which could not have been detected by earlier methods (Steel, 1971). Other cell lines had more complicated rearrangements: translocations, deletions, and insertions, many of which were identified by their fluorescence pattern. It is interesting that the abnormal chromosomes common in the human population, e.g., D/G and D/D translocations, have not been found in the cultured lines.

Each cell line appears to have specific marker chromosomes with distinctive banding patterns. It may therefore be possible to use fluorescent karyotyping as a method for monitoring cell cultures. Miller et al. (1971d) showed that a D98/AG line carried three of the same marker chromosomes, identified by fluorescence pattern, as did their HeLa cultures. They concluded that the D98 was a HeLa contaminant, supporting the biochemical evidence of Gartler (1967).

Studies in Other Species

Differential binding with quinacrine compounds has been observed in species other than man, including such diverse organisms as several plants (Caspersson et al., 1969a; Vosa, 1970a), various species of Drosophila (Vosa, 1970b; Ellison and Barr, 1971; Zuffardi et al., 1971), the marsupial rat kangaroo (Grewal et al., 1971), and the Chinese hamster (Caspersson, 1970f). In the mouse *Mus musculus,* all 20 pairs of chromosomes, which are telocentric, can be identified by their characteristic fluorescent banding patterns (Dev et al., 1971; Francke and Nesbitt, 1971), whereas only one pair could be identified before. This technique has been used to delineate a series of translocations and to assign linkage groups to 14 of the mouse chromosomes (Kouri et al., 1971; Miller et al., 1971b, 1971e, 1971f; Nesbitt and Francke, 1971).

Pearson and his associates (1971) examined a series of 27 mammalian species by the quinacrine fluorescence technique. The most striking finding was that only man, chimpanzee, and gorilla have intensely fluorescent regions on the autosomes and that only man and the gorilla have an intensely fluorescent Y. The chimpanzees studied had a small Y.

Interspecific Somatic Cell Hybrids

Identification of Specific Human Chromosomes. Caspersson and his associates (1971b) showed that certain chromosomes can be identified by their characteristic fluorescent banding patterns in somatic hybrid cells even when the mouse parent is a heteroploid line containing numerous biarmed chromosomes. Allderdice and her associates (1972) have extended this work to a large series of such hybrid lines and shown that every human chromosome can be identified against such a mouse background.

Application to Cytogenetic Mapping. i. Thymidine kinase and chromosome 17. When hybrids are produced between a mouse cell line deficient in thymidine kinase and human diploid cells, the viability of the hybrid cells in HAT medium is dependent on the presence of human thymidine kinase (Migeon et al., 1969) and a human E-group chromosome, number 17 or 18 (Matsuya et al., 1968; Migeon and Miller, 1968), which is therefore thought to carry the structural locus for thymidine kinase. This chromosome has been identified as a number 17 by analysis of its fluorescent banding patterns (Miller et al., 1971c). A possible translocation between human chromosome 17 and a mouse chromosome has been postulated on the basis of an acquired change in chromosome 17 in a similar man-mouse hybrid (Boone et al., 1972).

ii. Phosphoglycerate kinase and the long arm of the X. The use of the fluorescent banding analysis of the chromosomes in man-Syrian hamster hybrid cells in which the hamster parent is deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT) has made it possible to discover selective

retention of both parts of the human X chromosome in such hybrids in HAT selective medium, and to show that the phosphoglycerate kinase locus is on the long arm of the human X (Grzeschick et al., 1972).

iii. Lactic dehydrogenase A and chromosome 11. Boone et al. (1972) reported a positive correlation between the presence or absence of lactic dehydrogenase A (LDH-A) and that of human chromosome 11 in man-mouse hybrid cultures. In a culture positive for human LDH-A, but containing no normal chromosome 11, they observed one chromosome whose quinacrine fluorescent banding pattern was consistent with its being a number 11 which had undergone inversion. Shows (1972) studied similar man-mouse hybrids and reported that the lactic dehydrogenase A gene locus is linked to that of esterase A_4 but is not linked to loci for 13 other enzymes. This suggests that both the LDH-A and esterase A_4 loci are on chromosome 11.

iv. Isocitric dehydrogenase and chromosome 20. A tentative assignment of isocitric dehydrogenase to human chromosome 20 has been made by Boone et al. (1972).

Evaluation of Quinacrine Method

The development of the quinacrine fluorescence technique has revolutionized human cytogenetics. The ability to recognize each chromosome and to analyze even small reciprocal translocations permits a much more exact basis for defining new clinical syndromes, for genetic counseling, and for prenatal diagnosis. Since banding patterns are so specific, only a few good cells are required for routine work to analyze abnormalities other than mosaicism, and the karyotypes serve as permanent records.

The quinacrine fluorescence technique has certain disadvantages. The preparations must be examined using a fluorescence microscope. The cells must be photographed, since the brightness diminishes with time, and exposure times are long. Visual analysis is usually completed by preparing karyotypes from photographic prints, and care must be taken that these are of good quality.

Other Fluorochromes

No fluorochrome has been found which produces banding as satisfactory as that of quinacrine mustard or quinacrine. Acriflavine (Fig. 1) and proflavine (2,8-diaminoacridine sulfate) give banding patterns similar to those of quinacrine mustard (Caspersson et al., 1969a; Vosa, 1970a). A third acridine dye, Acranil (another diaminoacridine), appears to produce similar banding (Moscetti et al., 1971). Acridine orange gives only faint banding with *Trillium* chromosomes, but so does quinacrine (Caspersson et al., 1969b). It is thus possible that virtually all the acridine dyes produce similar banding patterns.

Ethidium bromide, a phenanthridium dye (Fig. 1), produces brightly

fluorescent chromosomes with no obvious banding or else a banding pattern which is the reverse of that produced by Q or QM (Caspersson et al., 1969b; Vosa, 1970a; Pearson et al., 1971).

MODIFIED GIEMSA STAINING METHODS

Characteristics of Cationic Dyes

Most of the nonfluorescent banding techniques involve the use of Giemsa stain. This stain contains the basic (cationic) dyes methylene blue and azure 1, or methylene azure, a somewhat variable mixture obtained by the oxidation of methylene blue and containing the similar compounds azure A, B, and C (Fig. 1). Giemsa also contains the acidic dye eosin, and this combination of dyes produces staining effects which are not exactly those produced by the individual dyes, for reasons which are not entirely clear. Under the usual conditions, Giemsa stains the nucleus and chromosomes metachromatically, producing a deep reddish-violet color, although its basic dye components are bluer than this. Such metachromatic staining occurs with many of these cationic dyes (including the fluorochromes), which undergo a major shift in their visible (and ultraviolet) light absorption to a lower wavelength upon being bound by polyelectrolytes such as DNA or synthetic polynucleotides (Steiner and Beers, 1959).

Methylene blue stains isolated DNA or intact nuclei metachromatically, producing an orange-red color. Orthochromatic (blue) staining occurs if the DNA in either preparation is denatured by heating to 88°-95° C in the presence of 4% formaldehyde, and metachromatic staining cannot be reinduced (Silha, 1966). The action of formaldehyde in stabilizing single-stranded DNA or synthetic polynucleotides is well known (Thomas and Berns, 1962).

Basic dyes bind to DNA by intercalation or by combining with the phosphate groups of DNA in definite stoichiometric proportions (Kurnick, 1950; Klein and Szirmai, 1963; Miura and Ohba, 1967). Intercalation requires the presence of native DNA. Single-stranded DNA binds less dye, as expected, and it fails to stain metachromatically, e.g., with methylene blue (Kurnick, 1950; Silha, 1966). The binding of histones to DNA also involves phosphate groups, and as many as half of these groups may be bound in this way. Thus, twice as much azure A will bind to DNA as to deoxyribonucleoprotein (DNP) (Klein and Szirmai, 1963) or intact nuclei (Szirmai and van der Linde, 1963). The interfering cationic groups of the nucleoprotein can be removed by acetylation, with resultant increase in the binding capacity of DNA for methylene blue (Kiefer et al., 1968). The binding of azure A to DNA is not influenced by added salts or fixation in ethanol, formaldehyde, or osmium tetroxide, but its binding to DNP or nuclei is increased by these measures, presumably because they tend to remove histones and make more phosphate groups in DNA available for dye binding (Stellwagen and Cole, 1969). Acetic acid-containing fixatives also remove histones (Dick and Johns, 1967).

Centromeric Heterochromatin Staining (*C Banding*)

Pardue and Gall (1970) observed that the centromeric regions of mouse chromosomes were deeply stained by Giemsa after treatment to denature the DNA of the chromosome, whereas the remainder of the chromosomes stained very faintly. Arrighi and Hsu (1971) adapted this method to human chromosomes. Fixed cells were treated successively with HCl to remove histones (and some other proteins), RNase to remove RNA, and NaOH to denature the DNA, and were then incubated in 2 × SSC (i.e., 0.3 *M* sodium chloride–0.03 *M* sodium citrate) for 20 hours before staining with Giemsa at a neutral pH. The only chromosome regions which stained deeply after this treatment were blocks at the centromeric region (Fig. 6). The secondary constriction regions on the long arm of chromosome 1 and 16 and probably 9 were deeply stained, along with the distal half of the Y. The Giemsa-stained blocks, which are sometimes called C bands, varied in size and location from chromosome to chromosome, and even the same chromosomes showed blocks of variable size (Craig-Holmes and Shaw, 1971). Because of this variation, and the small differences between chromosomes, the C-banding technique does not appear generally useful for chromosome identification. Claims for the identification of specific human

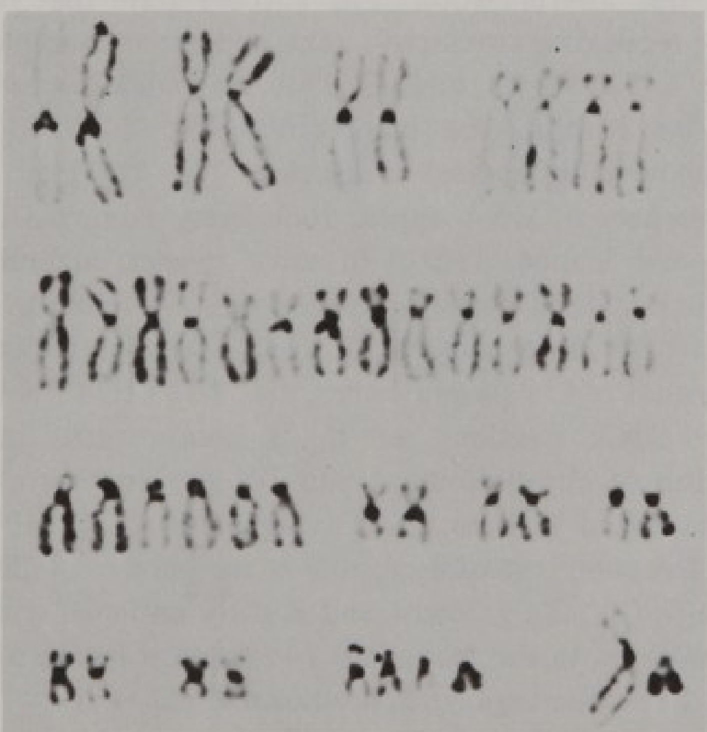

FIG. 6.–Karyotype of a metaphase cell from a male, stained by the centromeric heterochromatin (C-banding) method. (From Arrighi and Hsu, 1971. By permission of S. Karger, Basel.)

chromosomes within man-mouse hybrid cells by this technique (Chen and Ruddle, 1971) depend on using an independent and reliable method for identifying the chromosomes. It is potentially extremely valuable as a method for locating the centromeres of meiotic chromosomes, as illustrated in a recent study of a Y/autosome translocation (Caspersson et al., 1971c).

An interesting variation of the C-banding technique has been described by M. Bobrow et al. (personal communication), who treated chromosomes with Giemsa adjusted to pH 11 with dilute NaOH. They observed bright-red staining of the secondary constriction of chromosome 9, and less in other heterochromate regions such as the distal part of the Y and the short arms of the acrocentric chromosomes.

The deep staining of centromeric heterochromatin by Giemsa and the much weaker staining of other chromosomal regions after NaOH treatment have led to the suggestion that Giemsa stains only native DNA (Sumner et al., 1971a). This has been clearly established for one of the components of Giemsa, methylene blue (Silha, 1966), and fits with all that is known about basic dyes. Yunis et al. (1971) also assumed that Giemsa stains only native DNA and interpret as reassociated DNA those chromosomal regions that are darkly staining following treatment with NaOH and hot buffers. Small regions around each centromere and the distal portion of the long arm of the Y chromosome are most rapidly reassociated by this interpretation. The authors state that regions which are known to be late replicating reassociate next, and use as examples certain regions of chromosomes 4, 13, 16, 18, and 22. This is a somewhat dubious claim, since four of these five chromosome pairs cannot be identified without special techniques, which were not applied in this case.

Repeated sequences of DNA appear to be very common in all mammalian species (Britten and Kohne, 1968). In some species, including man (Corneo et al., 1970), this is at least partially in the form of satellite DNA, distinguished by its buoyant density. In general, repetitive DNA is located in the centromeric regions or at the ends of the chromosomes, but the labeled RNA complementary to one satellite DNA fraction, sat II, is concentrated in the secondary constriction region of chromosome 1 and 16, and another chromosome pair, probably 9 (Jones and Corneo, 1971). Saunders et al. (1972a) found that highly labeled RNA complementary to one of the human satellite DNA fractions isolated in an Ag^{+}–Cs_2SO_4 gradient and slightly enriched with respect to G-C bases was concentrated in the secondary constriction region of chromosome 9, but not at all in the similar region of chromosome 1.

Jones and Corneo (1971) observed differences in the degree of uptake of labeled sat II *c*RNA by the two chromosomes 1. This may indicate heritable polymorphism in the amount of sat II DNA at specific sites. Saunders et al. (1972a, 1972b) have separated several populations of repeated DNA sequences in human cells, prepared highly radioactive RNA complementary to some of

these (called sat *c*RNA), and localized them to specific chromosomal regions by utilizing in situ hybridization and autoradiography. They suggest that human repetitious DNA, unlike that of the mouse, is composed of a variety of DNA classes, and that mapping human chromosomes by nucleic acid hybridization appears feasible.

Giemsa Banding Techniques (G Banding)

Acetic-Saline-Giemsa (ASG). Sumner et al. (1971a) treated acetic alcohol-fixed preparations of metaphase chromosomes with 2 × SSC at 60° for 1 hour prior to staining with Giemsa at pH 6.8. This has been referred to as the ASG technique (acetic/saline/Giemsa). The banding patterns produced are very similar to those obtained with the quinacrine fluorescence technique (Fig. 7). Evans et al. (1971), who stained cells first with ASG and then with quinacrine, give a brief description of the banding pattern of each chromosome. Differences between quinacrine and Giemsa banding were observed in the secondary constriction regions of chromosomes 1 and 16, which are very faintly fluorescent but are intensely stained with Giemsa, chromosomes 11 and 12, which show a more pronounced nonstaining region in the proximal part of the long arm using the ASG method, and the Y, which stains intensely with quinacrine but not with Giemsa.

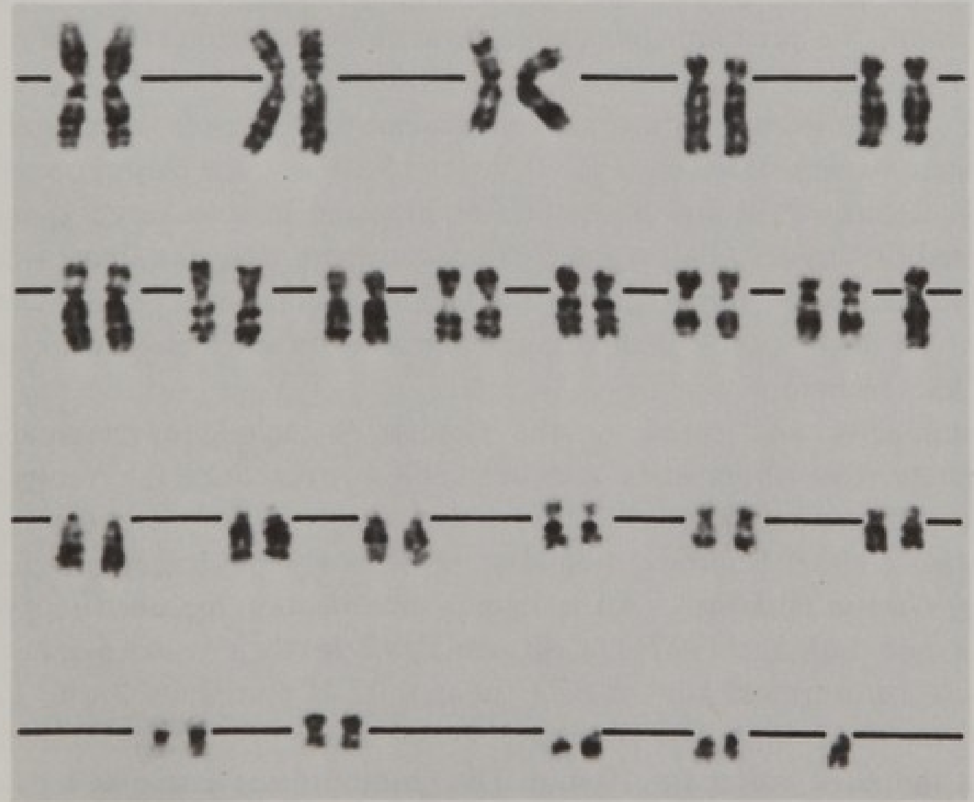

FIG. 7.–Karyotype of a metaphase cell stained by the ASG (acetic-saline-Giemsa) technique. (From Sumner, Evans, and Buckland, 1971. By permission of *Nature*, London.)

As with quinacrine fluorescent banding, more bands are seen with Giemsa banding techniques such as ASG when the chromosomes are in an extended state. The bands tend to be narrower than the corresponding quinacrine bands, and in contracted chromosomes more bands are seen by the Giemsa method. The use of excessive heat, e.g., flaming of fixative, during slide preparation prevents the formation of Giemsa bands.

Chromosome banding can be produced by other saline treatments. Dev et al. (1972a) have shown that shorter treatments at room temperature will work, and that removal of the divalent cations Ca^{2+} and Mg^{2+} plays a role.

Denaturation-Renaturation. Several techniques for producing banding throughout the chromosomes use treatment designed to denature DNA, followed by long incubation in hot salt solutions. Schnedl (1971a) follows pretreatment with .07 *N* NaOH for 90 sec with incubation for 24 hours in phosphate buffer (pH 6.8) at 59°. Drets and Shaw (1971) pretreat with .07 NaOH for 30 sec, then incubate in 12 × SSC at 65° for 60-72 hours.

Both these methods produce darkly staining centromeric and secondary constriction regions as in the centromeric heterochromatin technique. In contrast to other Giemsa banding techniques, the distal end of the Y is stained intensely by these methods.

Giemsa 9. Patil et al. (1971) found that differential staining of human chromosomes can be obtained simply by changing the pH of their Giemsa stain from the usual 6.8 to 9.0. The duration of staining at pH 9.0 was also important. After 1-2 min, the predominant staining was in the centromeric regions, with occasional staining of the secondary constriction regions of chromosomes 1 and 16. After 4-10 min, a banding pattern similar to that seen after quinacrine mustard staining was observed (Fig. 8). After 15-30 min, the chromosomes were uniformly stained. Patil and his associates prepared double karyotypes of 50 cells, stained first by the Giemsa 9 technique and then, after destaining with 70% ethanol, stained with quinacrine mustard. The major banding characteristics were identical in the two techniques, and the same differences were apparent as with the ASG technique.

The simplicity and speed of the Giemsa 9 technique recommend it. Unfortunately, few laboratories have been able to reproduce the results of the technique's originators, and until this problem is solved this method will not be able to replace either fluorescent banding or other Giemsa banding techniques.

Reverse Giemsa Banding. An intriguing modification has been reported by Dutrillaux and LeJeune (1971). Cells are fixed in their usual manner (with Carnoy–chloroform) and kept at 87°C in an 0.02 *M* phosphate buffer solution (pH 6.5) for 10-12 min. The slides are cooled slowly to 70° and stained with Giemsa in the same buffer for 10 min. The chromosomes have only a pale stain and are observed with phase contrast. The banding patterns appear to be the opposite of that resulting from the QM or the Giemsa methods described above;

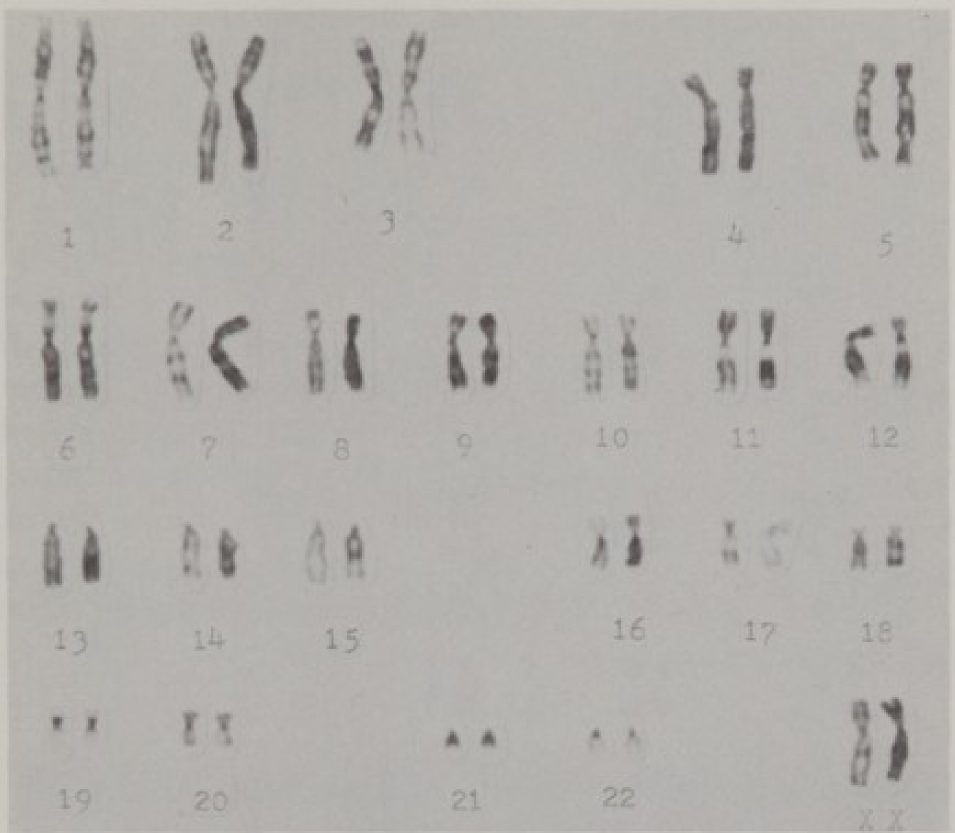

FIG. 8.–Karyotype of a metaphase cell stained by the Giemsa 9 technique. (Courtesy of H. A. Lubs, University of Colorado.)

i.e., those regions which take up stain and are bright with QM or dark with the other Giemsa methods do not take up stain and appear light by this method (Fig. 9) (Dutrillaux et al., 1972a). The Y chromosome was uniformly pale; the 2° constriction regions of chromosomes 1, 9, and 16 were not distinctive.

Dutrillaux and Lejeune state that they were able to identify the chromosome involved in 21 trisomy as that which had a pale staining proximal area, and were able to demonstrate a t(11p–;10q+) without difficulty. The method has been used to demonstrate a 21/21 translocation (Dutrillaux et al., 1972b) and a translocation involving chromosomes 9 and 21 (Rethoré et al., 1972). The method is somewhat disadvantageous since phase contrast is required. It is potentially valuable for study of translocations involving the ends of chromosomes since this is the only method in which most of these are known to appear darkly stained.

Proteolytic Enzyme Treatment. One of the most interesting modified Giemsa techniques involves pretreatment of fixed preparations with a proteolytic enzyme prior to staining. Dutrillaux and his associates (1971) described the results of a 3-6-min treatment with pronase. Seabright (1971) and Wang and Federoff (1972) published still more convincing evidence that enzymatic treatment could produce characteristic banding patterns which are indistinguish-

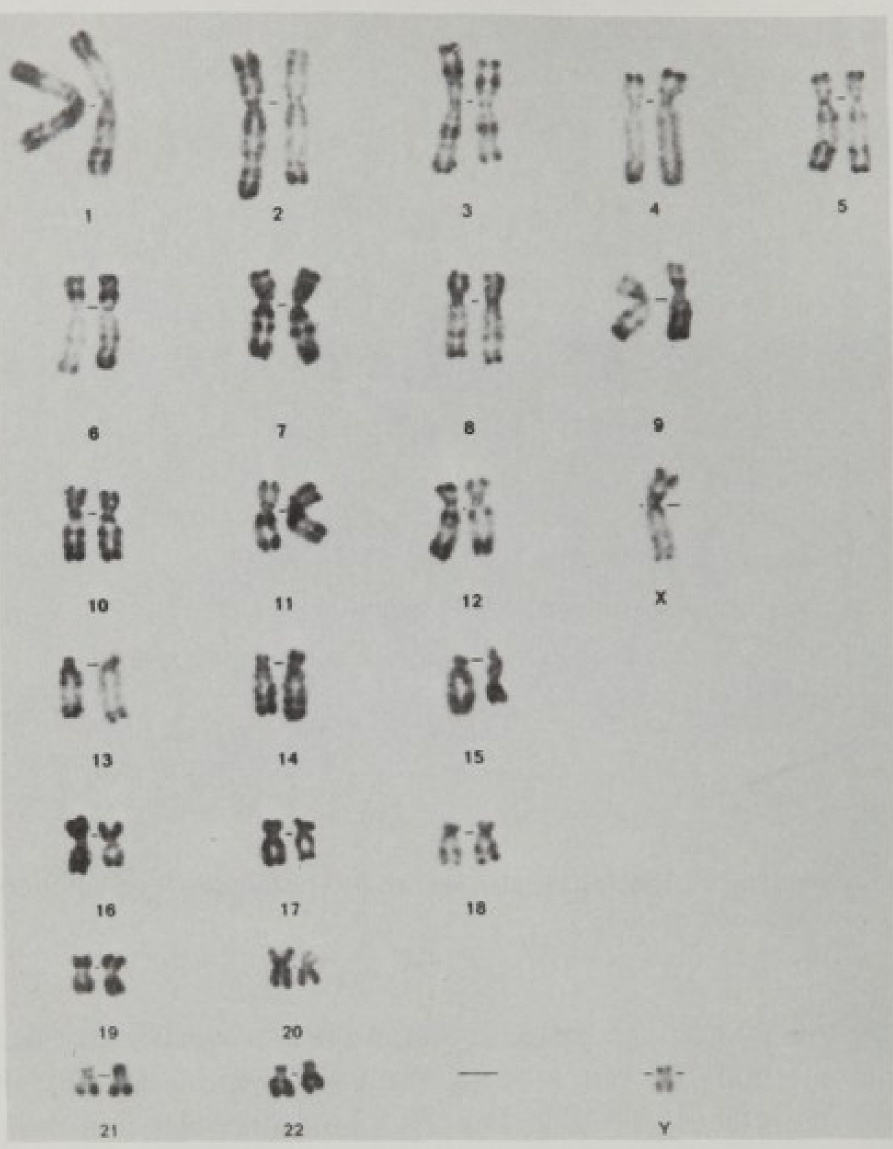

FIG. 9.–Karyotype of a metaphase cell stained by the technique of Dutrillaux and Lejeune, 1971. (By permission of Gautier-Villars, Paris.)

able from those produced by nonenzymatic methods of pretreatment (Fig. 12). Seabright (1971) used trypsin followed by a Leishman stain, whereas Wang and Federoff (1972) used trypsin, with or without added versene (EDTA), and stained with Giemsa. Very small translocations (the distal end of chromosome 1 to the long arm of 18), as well as 21 trisomy, could be demonstrated using this technique (Seabright, 1972). Highly purified crystalline trypsin works quite well, indicating that the effect is probably due to the enzyme molecule itself and not to a contaminant. Trypsin is not effective after its enzymatic activity is blocked by soybean inhibitor (Warburton, unpublished data). Trypsin-EDTA is more effective in salt solutions lacking the divalent cations Ca^{2+} and Mg^{2+} (Dev et al., 1972a).

FLUORESCENT ANTIBODY TECHNIQUE

Freeman and his associates (1971) have described a method which has the advantage of complete base specificity. They used fluorescein-tagged antinucleoside antibodies which are reactive only with single-stranded DNA and are directed against the individual purine or pyrimidine bases. Fixed metaphase chromosomes do not stain with these antibodies unless the chromosomal DNA is first denatured. After 1 hour in formamide at 65° the chromosomes are brilliantly fluorescent when stained with either antiguanosine (anti-G) or antiadenosine (anti-A) antibodies (Dev et al., 1972b). The banding appearance along the chromosomes stained with anti-A is remarkably similar to that seen with quinacrine staining (Fig. 10). The centromeric region of each chromosome, the secondary constriction regions of 1, 9, and 16, and the distal end of the Y do not react with either antibody after this treatment, presumably because these areas contain only double-stranded DNA.

Anti-A gives sharper banding than anti-G, and this suggests that differences in

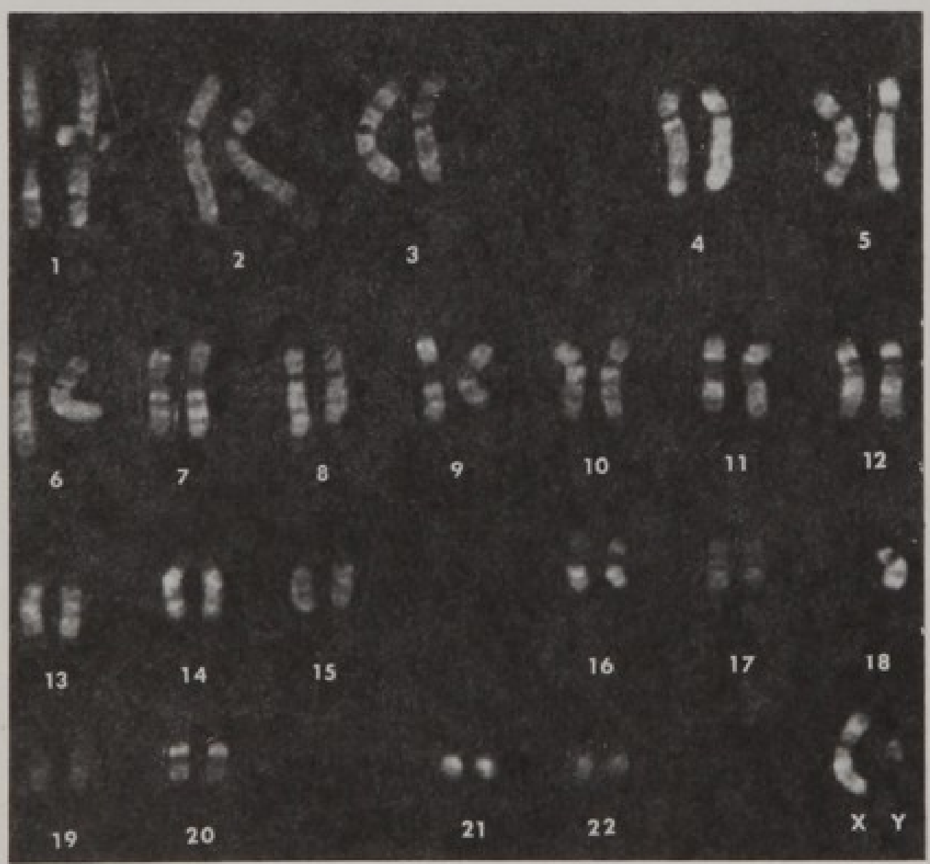

FIG. 10.–Karyotype prepared from a metaphase cell stained with fluorescein-tagged antiadenosine antibody specific for single-stranded DNA. Banding pattern similar to quinacrine. The centromeric regions, secondary constriction regions in number 1, 9, and 16, and the distal portion of the Y are all faintly stained. One number 18 is lying beneath a number 1. (Courtesy of V. G. Dev, Columbia University.)

base composition do play a role in the distinctive banding patterns of the chromosomes after a variety of treatments or staining procedures. This idea is supported by the production of a characteristic reverse banding pattern by fluorescein-tagged anticytidine after guanine bases in chromosomal DNA are destroyed by photooxidation (R. Schreck et al., personal communication).

RELATED CHARACTERISTICS

Time of DNA Replication

Caspersson and his associates (1968) noted, in the plant *Trillium*, that the chromosome region which is intensely fluorescent after QM staining is late replicating, and suggested that heterochromatin is generally more brightly fluorescent than euchromatin. Ganner and Evans (1971) correlated the intensity of fluorescence of human chromosome segments with the timing of DNA replication. There were significantly higher grain counts over each of seven more brightly fluorescent chromosomes or chromosome segments (selected from numbers 1, 13-15, and 17-22) than over a chromosome (or segment) of comparable size but lesser fluorescence. That is, most regions which show bright fluorescence are also late replicating (Fig. 11). However, not all late replicating regions are brightly fluorescent. The most notable example is the late-replicating inactive X chromosome in females, which has the same fluorescent pattern and intensity as the active X (Caspersson et al., 1971g; Ganner and Evans, 1971; Breg et al., 1972a). Autoradiography therefore remains the only method of identifying the inactive X chromosome at metaphase, other than the modified fixation method of Saksela and Moorhead (1962).

The late-replicating regions of chromosomes appear to represent several different categories on the basis of their various staining reactions. The secondary constriction regions of chromosomes 1, 9, and 16 and the centromeric region of number 22, which are all late replicating, are faintly stained by quinacrine. The secondary constrictions of chromosomes 1 and 16 and the centromere of 22 are also darkly stained with Giemsa whereas the secondary constriction region of 9 is not. Other late-replicating regions such as the distal end of the Y and the satellites of one or more D-group chromosomes have intense fluorescence but average Giemsa staining (Ganner and Evans, 1971; Breg et al., 1972a). An example is seen in Fig. 11, chromosome 14.

It is interesting that although chromosomes 19 and 20 cannot be distinguished from one another on the basis of their terminal DNA replication patterns (de Capoa et al., 1967; Giannelli and Howlett, 1971), once they have been identified by their fluorescent banding pattern it is easy to demonstrate the significantly later replication of chromosome 20 than chromosome 19 (Ganner and Evans, 1971). Combined measurements of DNA content and grain counts (as estimates of late DNA synthesis) led Giannelli and Howlett (1971) to

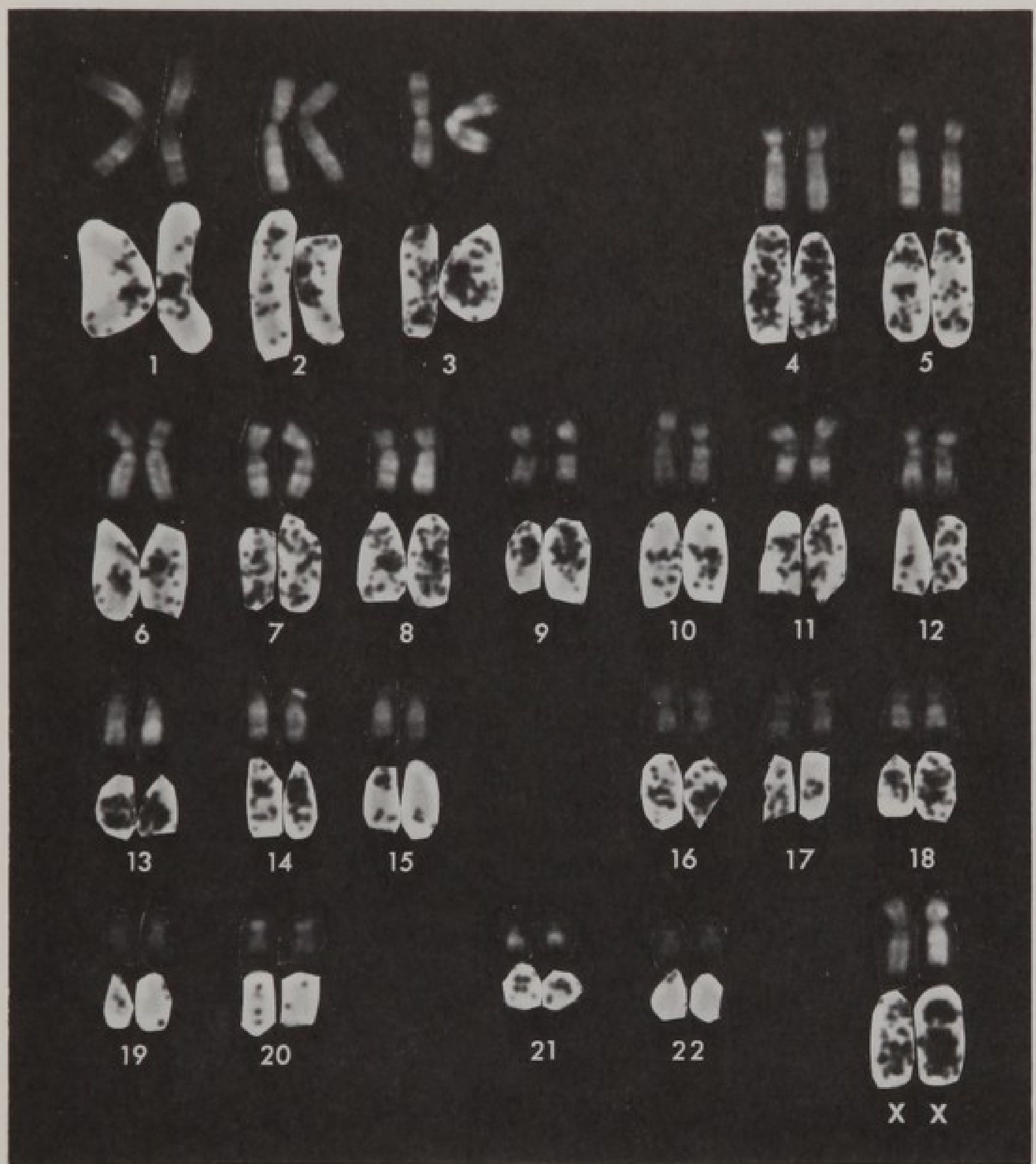

FIG. 11.–Double karyotype of a normal female. Chromosomes identified by quinacrine fluorescence pattern. Cultures grown in the presence of tritiated thymidine, and autoradiography performed to demonstrate the terminal DNA replication pattern. (Preparation courtesy of P. W. Allderdice, Columbia University.)

conclude that the pair of F-group chromosomes with more DNA is later replicating. They called this pair number 19, but in accordance with the system of nomenclature adopted at the Paris Conference (1971), this will have to be called pair number 20 despite its apparently slightly greater DNA content.

Now that various banding techniques have been developed which permit accurate identification of every chromosome, autoradiographic analysis of

terminal DNA synthesis should no longer be used as a method of chromosome identification. Already several examples of misidentification by autoradiography have become apparent (see p. 10), and the inherent limitations of this technique need no longer be disregarded. Autoradiography remains, however, the most useful method for identifying the inactive X in metaphase cells and is of extreme value in analyzing X-autosome translocations (Buckton et al., 1971).

Secondary Constrictions

In general, the secondary constrictions which are sometimes seen on various chromosomes in the complement (Ferguson-Smith et al., 1962) coincide with regions of negative fluorescence, although not all regions of negative fluorescence show secondary constrictions. Saksela and Moorhead (1962) produced a "fuzzy" appearance of the secondary constriction of chromosomes 1, 9, and 16, and of the entire late-replicating X, by using a modified fixative. Of these regions, only the X is even moderately brightly fluorescent after quinacrine staining. Palmer (1970) observed marked chromatid attenuation, or constriction, following growth of cells in the presence of 5-bromodeoxyuridine and use of the same fixative. In addition to the usual constrictions on chromosomes 1, 9, and 16, others were present on chromosomes 13 and 14, and elsewhere. The most striking feature was the attenuated distal segment of the long arm of the Y chromosome, a region which usually is intensely fluorescent after quinacrine staining.

COMPARISON OF CHROMOSOME BANDING TECHNIQUES

Characteristics of the various techniques discussed above have been summarized (see Table 1) in order to facilitate comparisons. The positively and negatively stained bands appear to be in corresponding locations, with a few exceptions, on chromosomes stained by the quinacrine fluorescence or Giemsa banding techniques. This is illustrated in Fig. 12 for cells stained with quinacrine mustard or with Giemsa after treatment with trypsin. Discrepancies among the methods are pointed out in the table. The most strking differences in staining characteristics are those of the Y chromosome, in which the distal region of the long arm is intensely stained by quinacrine, darkly stained by Giemsa using the centromeric heterochromatin technique, moderately stained by the various Giemsa banding methods, and almost nonstained by fluorescein-tagged antiadenosine (Fig. 13).

Identification of chromosomes by either the ASG or the trypsin technique has been carried out in a variety of laboratories with good results, although with less consistency than the quinacrine technique, perhaps because only the latter requires no pretreatment of the chromosomes. If the Giemsa banding technique fails, it is often possible to stain with quinacrine after the Giemsa stain is

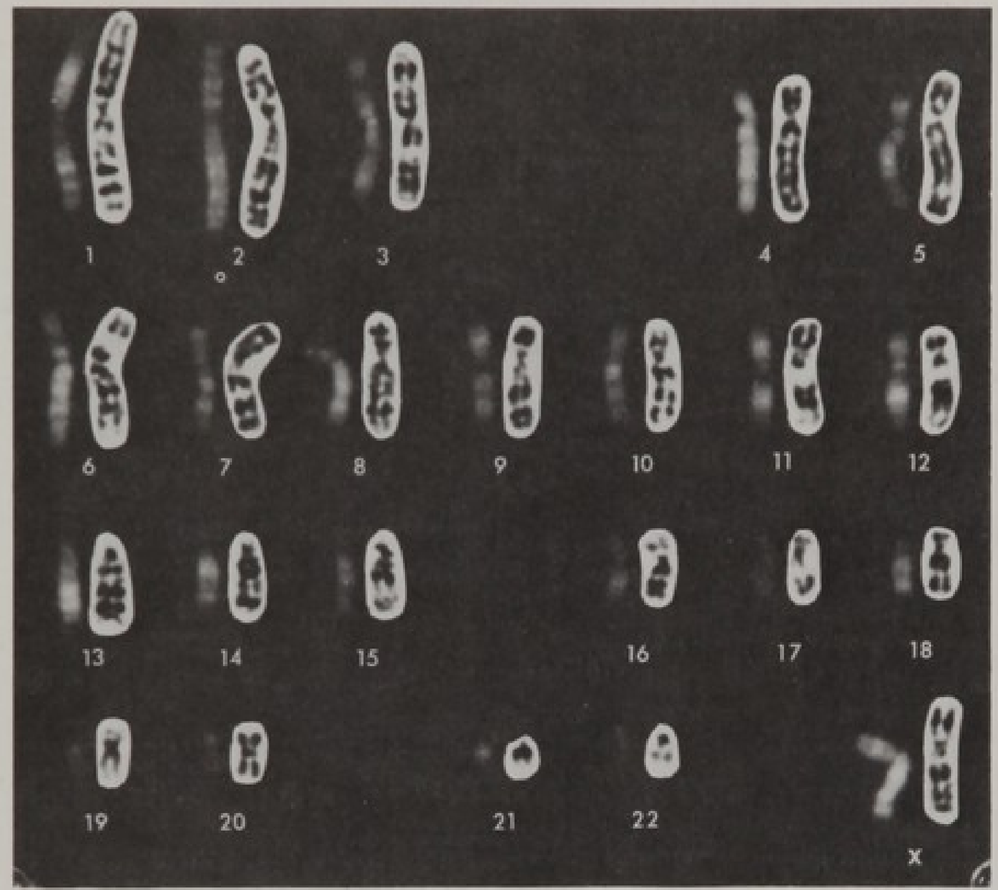

FIG. 12.–Composite karyotype arranged to permit comparison of banding patterns of the chromosomes in a cell stained with quinacrine mustard (*left*) and one stained with Giemsa following treatment with trypsin (*right*). (Fluorescent cell courtesy of P. W. Allderdice, Columbia University.)

removed with methanol. The quinacrine pattern appears unchanged after either ASG or trypsin treatments, although some secondary constriction areas may be less dully fluorescent (Evans et al., 1971) and the Y may be less intense. It is difficult to obtain good banding using the ASG or trypsin techniques after quinacrine staining.

Several of the techniques are not as suitable for chromosome identification but are useful in specific situations. The reverse Giemsa banding technique, which produces very palely stained chromosomes requiring phase contrast microscopy, is particularly suitable for investigation of abnormalities of the distal regions of the chromosomes. The centromeric heterochromatin staining technique, which makes it possible to locate centromeres unambiguously, is particularly useful in analyzing meiotic chromosome configurations. The autoradiographic technique is primarily useful in the identification of late replicating X chromosomes and in studies of X-autosome translocations. The antinucleoside antibody technique should provide important insights into chromosome organization, but it is not recommended for routine use, since preparation of the antisera is arduous and the results, in terms of banding,

TABLE 1.–*Comparison of Chromosome Banding Techniques*

	Technique (Slides usually fixed in 3:1 methanol:acetic acid, after hypotonic treatment with 0.07 *N* KCl)	Over-all banding pattern common to Q and G is present
Q banding	Stain 10 min with aqueous quinacrine or quinacrine mustard. Fluorescence microscopy	Yes
C banding	†0.2 *N* HCl, 30 min; RNase 37°, 1 hr; 0.2 *N* NaOH, 2 min; 10 hr in 2 × SSC at 65°; Giemsa stain	No
*c*RNA reannealing	As for C banding, but reannealed to RNA complementary to:	
	1. Satellite II DNA, density 1.693g/cm³ (Ag^{+}–Cs_2SO_4)	No
	2. Satellite DNA, density 1.703g/cm³ (Ag^{+}–Cs_2SO_4, pH 9.2)	?
G banding		
With ASG	1 hr in 2 × SSC at 65°; Giemsa stain	Yes
With Giemsa 9	10 min in Giemsa buffered to pH 9.0	Yes
With denaturation-renaturation	0.07 *N* NaOH for 30-90 sec Schnedl: 24 hr at 59° in phosphate buffer, pH 6.8 Drets and Shaw: 72 hr at 65° in 12 × SSC Giemsa stain	Yes
With trypsin	0.5% trypsin–EDTA (Gibco) for 1-2 min; Giemsa stain	Yes
R banding	‡0.02 *M* phosphate buffer, pH 6.5, at 87° for 10 min; cool slowly to 70°; Giemsa stain	Opposite pattern
Anti-A	1 hr in 95% formamide at 65°; rabbit adenosine for 45 min; fluorescein-tagged sheep-antirabbit GG for 45 min	Yes
Terminal DNA replication	³H autoradiography	No

* +, positive; ±, variable; ?, inadequate information; –, negative.

† Fixed in 50% acetic acid.

‡ Fixed in Carnoy's with chloroform.

Specific banding features*						
Secondary constrictions of 1 and 16	Secondary constriction of 9	Centromeres	Distal end of Y, polymorphic markers on 3, 4, and acrocentrics	Inactive X is distinguished	Figure	References
–	–	–	++	No	2	Caspersson, Lomakka and Zech (1971)
+	+	+	+	No	6	Arrighi and Hsu (1971)
+	+	?	?	No		Jones and Corneo (1971)
–	+?	?	?	No		Saunders et al. (1972a)
+	±	±	±	No	7	Sumner, Evans, and Buckland (1971)
+	±	±	±	No	8	Patil, Merrick, and Lubs (1971)
+	+	+	+	No		Schnedl (1971a); Drets and Shaw (1971)
+	±	±	–	No	12	Seabright (1971)
–	±	+	–	No	9	Dutrillaux and Lejeune (1971)
–	–	–	–	No	10	Dev et al. (1972)
+	+	?	+	Yes	11	Miller (1970)

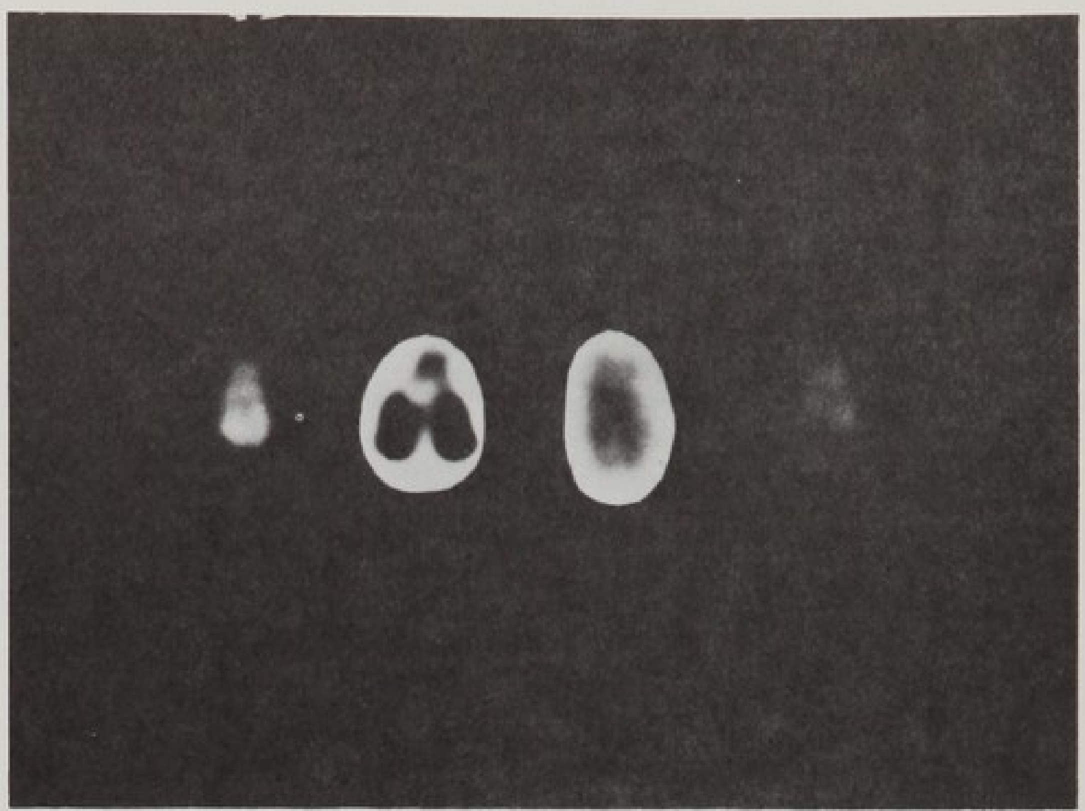

FIG. 13.–Appearance of the Y chromosome stained as follows (*left to right*): quinacrine mustard, Giemsa after NaOH denaturation, Giemsa after trypsin, and fluorescein-tagged antiadenosine.

appear to be similar to those obtained with the more readily available quinacrine or quinacrine mustard.

In situ molecular hybridization, on the other hand, is a technique which promises to be increasingly useful in mapping the locations of particular types of DNA or perhaps even specific genes. In *Drosophila melanogaster,* the genes coding for 5*s*RNA have been localized by this technique to region 56 EF of the right arm of chromosome 2 (Wimber and Steffensen, 1970), whereas the *t*RNA genes are scattered over more than 60 sites (Steffensen and Wimber, 1971). Such studies are more readily performed in an organism, like *Drosophila,* with giant polytene chromosomes, but they should provide a great deal of information about the chromosomes of man, especially when used in conjunction with the new staining techniques.

MECHANISMS OF CHROMOSOME BANDING

The underlying cause of the banded appearance of chromosomes after any one of the treatments discussed in this review is unknown. There are striking similarities in the banding patterns produced by quinacrine or quinacrine mustard, certain other fluorochromes, various Giemsa banding techniques, and fluorescein-tagged antibodies to bovine serum albumin-conjugated adenosine.

Information gathered from studies involving any of these methods should thus be useful in understanding the mechanism of production of chromosomal banding. Conversely, any proposed mechanism must account for the presence of a common pattern in all these methods.

Role of DNA

All the cationic dyes used in the various banking techniques react almost solely with DNA. It is therefore not surprising that DNase abolishes QM banding whereas removal of RNA or histones has no effect (Comings, 1971; de la Chapelle et al., 1971). Variations in total DNA content per unit length of chromosome (measured by ultraviolet absorption or Feulgen photometry) cannot be solely responsible for the banding patterns, because they are very poorly correlated with the intensity of QM fluorescence along the chromosomes in both plants and animals (Caspersson et al., 1969 a, 1970f). On the other hand, variations in the type of DNA may be involved.

Base composition. Caspersson and his co-workers (1969a, 1969b) studied various classes of fluorescent compounds in a search for a substance with nucleic acid base specificity, in the hope of delineating segments of chromosomal DNA that are enriched with respect to a given base. Actinomycin D and chloroquine, which bind specifically to guanine, and Nogalomycin, which binds to adenine or thymine, produced such faint fluorescence of the chromosomes that they could not be used with the available systems of recording intensity of fluorescence. The alkylating agent quinacrine mustard, on the other hand, produced bright fluorescence, with marked regional differences in intensity. Because of the evidence that alkylating agents are much more reactive with the N-7 position of guanine than with sites on the other DNA bases, Caspersson et al. (1969a) suggested that the QM banding patterns might be the result of preferential binding of QM to the G+C rich regions.

Since Q and QM usually give identical banding patterns, this explanation implies that quinacrine too binds preferentially to G+C rich regions or that its fluorescence is enhanced in such regions. However, studies with polynucleotides indicate that preferential binding of quinacrine does not occur (O'Brien et al., 1966), and several bits of evidence suggest that G+C-rich regions do not enhance quinacrine fluoresence. Weisblum and de Haseth (1972) have shown in vitro that DNA rich in A+T enhances quinacrine fluorescence, whereas DNA rich in guanine actually quenches fluorescence. Ellison and Barr (1972) have shown that the area of intense quinacrine fluorescence on the Y chromosome of the dipteran insect *Samoaia leonensis* is late replicating and very rich in A+T, containing virtually no G+C. The same may be true of the intensely quinacrine fluorescent heterochromatic regions of the Y chromosome of *Drosophila melanogaster* (Blumenfeld and Forrest, 1971; Vosa, 1970b; Ellison and Barr, 1971). Bands of bright quinacrine fluorescence thus probably mark A+T-rich regions. This idea is

supported by the similarity of the banding patterns produced by quinacrine and by fluorescein-tagged antiadenosine antibodies (Dev et al., 1972b).

Specific bases do not have the same effect on all acridine dyes. For example, the fluorescence of acridine orange is markedly quenched by the synthetic polynucleotide polyriboadenylic acid (Steiner and Beers, 1961). Differential enhancement of ethidium bromide fluorescence by binding to G+C-rich regions would provide an explanation for the paradoxical finding that the banding pattern produced by ethidium bromide tends to be the reverse of that produced by quinacrine (Caspersson et al., 1969b; Vosa, 1970a; Pearson et al., 1971) despite the fact that the two dyes bind competitively and probably to the same sites on DNA (Peacocke and Skerrett, 1956; Waring, 1965; LePecq and Paoletti, 1967). However, the marked increase in the intensity of fluorescence of ethidium bromide which occurs on binding to DNA does not appear to be base specific (LePecq and Paoletti, 1967).

In situ molecular hybridization with highly radioactive RNA complementary to specific fractions of human DNA, followed by autoradiography to localize the hybrid molecules, has led to the discovery that highly repetitious DNA is scattered along all the human chromosomes, although concentrated in the centromeric and telomeric regions (Arrighi et al., 1971). Available information is inadequate to determine whether the localization of highly repetitious DNA matches any of the common banding patterns. However, the secondary constriction regions of chromosomes 1 and 16 contain a more specific highly repetitious DNA fraction which is different from the one found in the corresponding region of number 9 (Saunders et al., 1972a,b). That of chromosome 9 has a slightly higher G+C content than the genome as a whole.

Native vs. denatured DNA. Lerman (1963) and Weisblum and de Haseth (1972) have shown in in vitro studies that quinacrine fluorescence is largely dependent on the presence of double-stranded DNA. Partial denaturation may influence quinacrine fluorescent banding patterns, but the evidence for this is conflicting. Gagné et al. (1971) claim that after treatment of fixed human chromosomes with 0.07 *M* NaOH, followed by heating in SSC, only the usually brightly quinacrine fluorescent regions continue to be fluorescent. Similar results have been reported by de la Chapelle et al., (1971) in the chromosomes of man as well as in the mouse and *Microtus agrestus.* They note that after such treatment QM fluorescence becomes intense in regions which contain rapidly reannealing DNA, as shown by brilliant green fluorescence with acridine orange (AO) and very faint elsewhere. They concluded that intense fluorescence is confined to regions of repetitious DNA. Pearson and Bobrow (personal communication) obtained contrasting results with human chromosomes, noting bright-green fluorescence, after exposure to 2 × SSC for 1 hour at 60°C and AO staining, only in regions which do not fluoresce brightly with quinacrine. The remainder of each chromosome showed dull-red fluorescence. Comings (1971),

on the other hand, has observed no effect of denaturation (indicated by red fluorescence of AO) on QM banding patterns in a mammal, the Indian muntjac. Ellison and Barr (1972) also see no effect in an insect of the *Drosophila* family, *Samoaia leonensis.*

Some of the discrepancies in these studies may be the result of very rapid renaturation of chromosomal DNA in situ. If DNA is denatured by NaOH or by heating in SSC or formamide, in the absence of formaldehyde, renaturation takes place and there is virtually no increase in the proportion of single-stranded DNA in *Drosophila* polytene chromosomes (Nash and Plaut, 1964) or intact mammalian nuclei (Rigler et al., 1969) as measured by the amount of red fluorescence after acridine orange staining.

Differential denaturation of DNAs of different base composition may provide an explanation of the reverse banding pattern observed by Dutrillaux and Lejeune (1971) when they heated metaphase spreads to 87°C in phosphate buffer of low ionic strength prior to staining with Giemsa. At this temperature, only A+T-rich regions can undergo denaturation, but GC pairs remain unaffected. At low ionic strength, renaturation does not readily occur. The net result would be a partial denaturation of A+T-rich regions. In view of the much greater intensity of staining of native DNA with basic dyes (Kurnick, 1950; Silha, 1966), one would then expect a reverse Giemsa banding pattern, for reasons now to be outlined.

Role of Protein

Only about half the DNA in intact chromosomes, or chromatin, is bound to protein. The unprotected DNA can be broken down by DNase, or fully protected against DNase by combining with polylysine (Clark and Felsenfeld, 1971). Chromosomal protein blocks the binding of some basic dyes to DNA, as discussed earlier, and it is therefore possible that the banding patterns observed after staining with fluorochromes or other basic dyes reflect to some extent the natural pattern of protein binding.

Acidic proteins are only about half as abundant in heterochromatin as in euchromatin without a corresponding increase in histone proteins (Frenster, 1965). This might account for the generally greater intensity of staining of heterochromatin, since proteins block dye-binding sites. The removal of proteins should enhance the staining of chromosomes by making more phosphate groups available (Szirmai and van der Linde, 1963; Angerer and Moudrianakis, 1972). Although the removal of histones has no effect on fluorescent banding patterns (Caspersson et al., 1969a; Comings, 1971; de la Chapelle et al., 1971), it may be important in the production of Giemsa G banding, which usually involves treatment of the chromosomes either with hot buffers whose ionic strength is sufficient to remove much of the protein or with proteolytic enzymes.

Treatment of fixed chromosomes with trypsin prior to Giemsa staining leads to very clear G banding (Seabright, 1971; Wang and Federoff, 1972). This suggests that the removal of one or more classes of protein is necessary for Giemsa banding. The removal of acidic proteins may occur in G-banding techniques which involve the use of NaOH or of Giemsa at a very alkaline pH, but the importance of this is unclear.

Although the removal of proteins appears to be important in permitting the development of chromosomal G banding, the underlying mechanism is not obvious. Since some of the G-banding treatments are rather short and mild, it seems likely that they succeed in removing only the proteins which are most weakly bound, e.g., the lysine-rich histone fraction f1, which is removed by considerably lower salt concentrations than the remaining histone fractions or nonhistone proteins (Akinrimisi et al., 1965; Ilyin and Georgiev, 1969). That is, differential binding of protein might be involved. Quinacrine and similar fluorochromes would presumably be able to displace the weakly bound proteins directly. Chromosome regions from which protein is released will have twice as many free phosphate groups and will therefore stain more deeply with Giemsa or other basic dyes–perhaps much more deeply, because of the stacking tendency of these dyes (Stone and Bradley, 1961). The remaining problem is why the proteins are more easily released from certain chromosomal regions than from others. Perhaps this is related to the type of purine or pyrimidine bases which are present. Polylysine, whose binding to DNA is essentially the same as that of the very lysine-rich histone fraction 1b (Akinrimisi et al., 1965), has a selective affinity for A+T-rich regions. Polyarginine exhibits a marked preference for the homopolymer polydG:dC, although only a slight preference for DNA only slightly enriched with respect to G+C (Leng and Felsenfeld, 1966). Base composition may thus account, directly or indirectly, for the fluorescent and the Giemsa banding patterns of the chromosomes.

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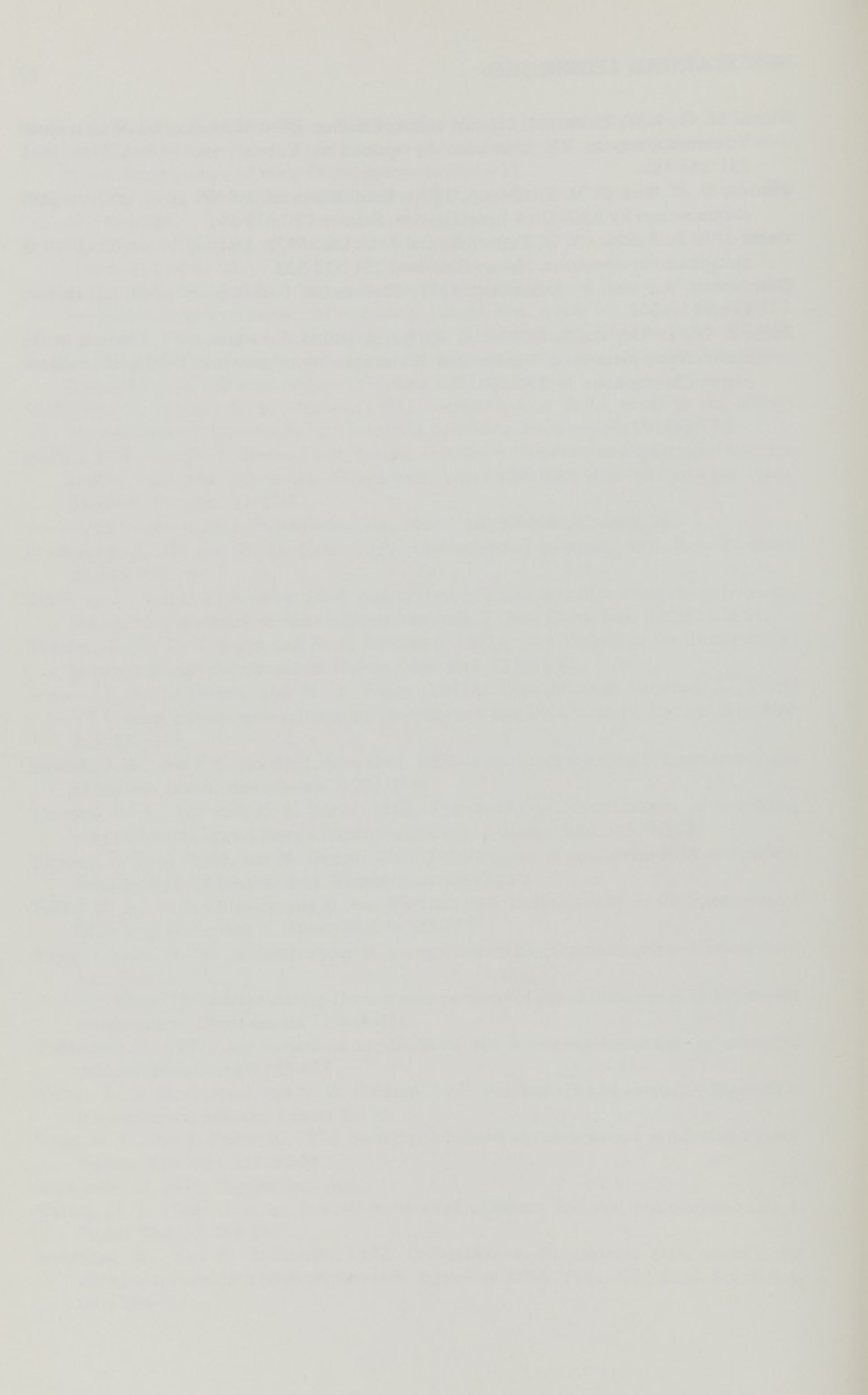

CHAPTER 2

The Genetics of Early Human Development

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Major advances in recent years have brought the earliest stages of animal and human development under laboratory control. A great deal of information from animals can now be usefully applied to the investigation of our own species, one example being the maturation of oocytes for studies on meiosis and fertilization. The fertilization of human oocytes is now under a fair degree of control, a step barely contemplated a few years ago. This has led to the development of methods needed to grow human embryos through the preimplantation stages of development. In this review, we wish to outline some studies in animals and man that are relevant to our understanding of the earliest stages of human development. We believe that a fuller understanding of these early stages will have important physiological, genetic, and clinical consequences, and we hope to illustrate some of these opportunities in this chapter.

Methods for analyzing the factors involved in the early differentiation of mammalian embryos have increased considerably in scope in recent years. Advances include the application of more refined methods for inducing controlled ovulation in animals and man, the rapid improvement of tissue culture techniques, the development of experimental embryology and more sensitive methods of biochemical analysis. Limitations are still imposed by the difficulty in obtaining large numbers of embryos, their small size, and the lack of suitable gene markers in early development. Analyses of preimplantation embryos began some years ago using techniques such as the destruction of individual blastomeres in cleaving embryos, or experimental treatments to alter their chromosome constitution. The number of experiments is now so numerous that it is impossible to quote them all. We will of necessity have to be selective, and refer to papers concerned with the genetics of early development, or to those of some immediate relevance to studies on human preimplantation development.

OOGENESIS AND FOLLICULAR GROWTH

Growth of the Ovary and Oocyte

In man, the region of the genital ridge is distinguishable in fetuses at about 30 days after ovulation (van Wagenen and Simpson, 1965). Rapid cell division and an increase in cell size are followed by the differentiation of an outer cortex and an inner medulla. Primordial germ cells are reported to arise in the endoderm in mammals (see review by Brambell, 1956), although more recent evidence suggests that they may originate in the primitive streak (Chretien, 1966). Primordial germ cells migrate into the presumptive gonad before 30 days postovulation (van Wagenen and Simpson, 1965). In female fetuses the primordial germ cells multiply as they differentiate into oogonia in the cortex. The movement of human oogonia and their mitotic divisions have been studied

in vitro (Blandau, 1969). The transformation of the oogonia into oocytes as represented by the first stages of meiotic prophase occurs between 3 and 7 months postconception in the fetal ovary (van Wagenen and Simpson, 1965). These authors, and others studying animal fetuses (Peters, 1970), have noted the presence of gradients in the development of oocytes at this time. The most advanced oocytes are always found near the corticomedullary boundary, the less advanced oocytes lying deeper in the cortex. Oocytes in the fetal ovary thus enter the first stage of meiosis in waves between the fourth and seventh months of fetal life. Synaptinemal complexes are detected in oocytes at this time (Baker and Franchi, 1967a, 1967b; Baker, 1971). The oocytes then begin the prolonged period known as dictyotene, which is distinctively characterized by the presence of a nucleus known as the germinal vesicle. The suspension of the meiotic process in this prolonged nucleated stage may be due to the influence of the granulosa cells which enclose the egg in early diplotene (Ohno and Smith, 1964). Meiosis and the formation of the germinal vesicle in the living human oocyte have been observed in culture (Blandau, 1969).

The oocyte remains in dictyotene until just before ovulation in the adult. Oocytes formed in the fetal ovary are now generally considered to be the sole source of oocytes in adult life. Various studies in mammals have indicated that oocytes are not formed in adult life; one approach has been to determine when DNA synthesis occurs in primary oocytes. The only period so far identified has been during the meiotic stage in the fetal ovary, and oocytes labeled with DNA precursors during this period are conserved until late in life (Sirlin and Edwards, 1959; Rudkin and Griech, 1962; Lima de Faria and Borum, 1962; Peters et al., 1965; Kennelly and Foote, 1966).

In the adult ovary, the oocyte enlarges some time before ovulation, and later follicular enlargement produces an early Graafian follicle (Pincus, 1965). Development to an early Graafian follicle is independent of the pituitary since it also occurs in hypophysectomized females (Zuckerman, 1962), although recent studies using antibodies against gonadotropins have indicated that these hormones are needed during the early stages of follicular growth (Lunenfeld and Eshkol, 1970). The number of follicles in the ovary is greatly reduced by atresia at various times during their formation, in puberty and during adult life (Ingram, 1962; Peters, 1970). Later follicular growth is certainly stimulated by follicle-stimulating hormone (FSH) released by the pituitary early in each menstrual cycle; several follicles enlarge although most of them became atretic and, in man, usually only one oocyte is ovulated. Estimates of the "transit time" of follicles through their various stages of growth have been obtained for mice (Pedersen, 1970). In most mammals, including man, the end of the dictyotene stage and the final maturation of the oocyte and ovulation itself are triggered by a midcycle surge of luteinizing hormone (LH) also secreted by the pituitary. The germinal vesicle regresses, the first meiotic division is completed with the

extrusion of the first polar body, and the second meiotic division proceeds to metaphase.

Details of the fine structure of human ovarian oocytes have been recorded by Baca and Zamboni (1967) and others. The oocyte is ovulated at this stage, i.e., while arrested in metaphase II, and remains in this stage until fertilization. Almost simultaneously with sperm entry into the oocyte, meiosis II is resumed at anaphase separation. At telophase, the second polar body is emitted from the egg. Details of human oocytes flushed from the oviducts are summarized by Mastroianni and Noriega (1970).

Stimulation of Follicular Development

Follicular growth can be induced by injecting gonadotropin into the female. The exogenous hormones replace those secreted endogenously, and large numbers of follicles can be stimulated into growth; the ovulation of large numbers of eggs after hormone treatment is known as superovulation. The normal rate of follicular atresia is evidently reduced by the availability of the exogenous gonadotropins, thus resulting in a greater number of oocytes completing their maturation and being ovulated. During these treatments, a follicular-stimulating preparation, usually human pituitary gonadotropin (HPG), human menopausal gonadotropin (HMG), or pregnant mare's serum (PMS), is given first, followed by a preparation containing luteinizing activity–usually human chorionic gonadotropin (HCG). The ratio of FSH to LH in different preparations influences the response to treatment, for an enhanced response was obtained in preparations with a higher proportion of LH (Bertrand et al., 1972). In animals, progression of the oocytes through the postdictyate stages after such treatments is very regular and can be timed with considerable accuracy following the injection of HCG (Chang, 1955a; Edwards and Gates, 1959).

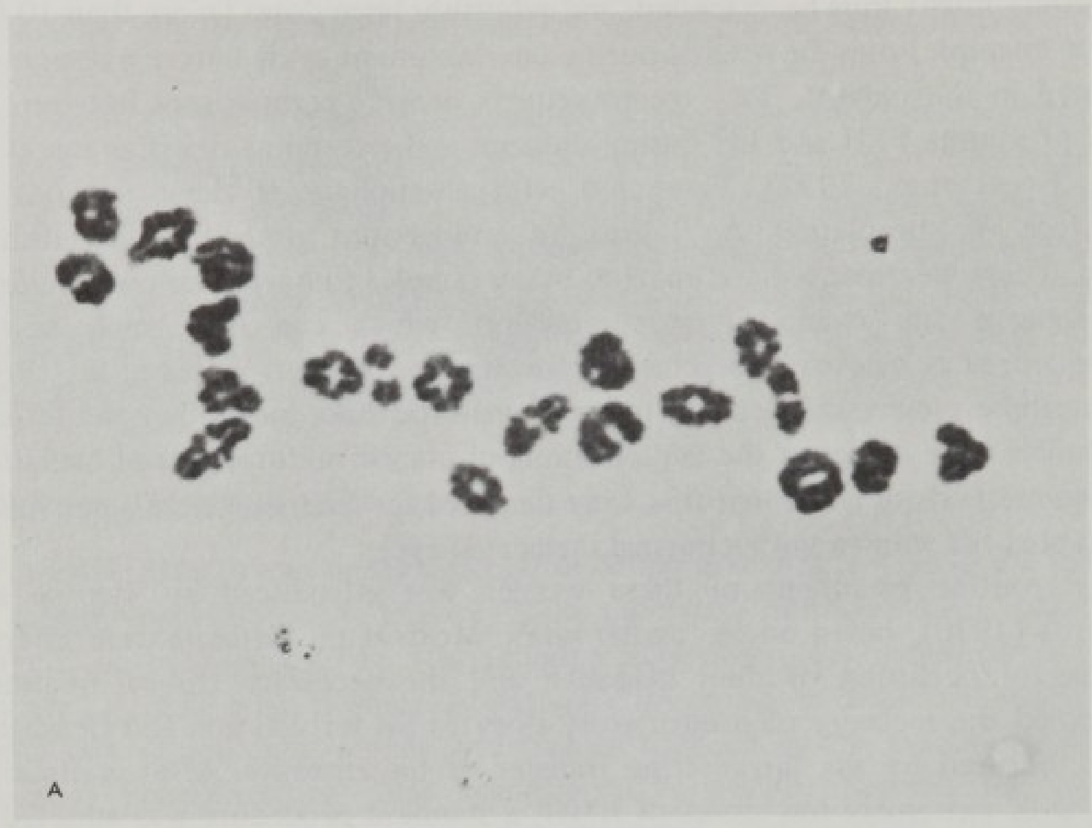
Endocrine methods for the induction of follicular maturation and ovulation have been developed for the treatment of human amenorrhea or oligomenorrhea (Gemzell, 1967; Brown et al., 1969; Crooke, 1969; Lunenfeld, 1969; Gemzell and Johansson, 1971). Treatments vary considerably among hospitals and doctors, although the rationale is similar. Injections of HMG, HPG, PMS, or clomiphene (a compound with estrogenic and antiestrogenic properties that stimulates release of endogenous gonadotropins) are used to stimulate the growth of ovarian follicles. Data from the treatment of amenorrheic women indicate that follicles require 10 days to grow into ovulatory follicles (Gemzell and Johansson, 1971; Bertrand et al., 1972). The response is often monitored by the excretion of urinary estrogens, which serves both as a guide to the response to treatment and a warning of the dangers of overstimulation.

At a predetermined time, or when urinary estrogens reach a particular level, one or more injections of HCG are given to induce ovulation. The treatment

with HMG might thus be extended, and relatively large doses (more than 10,000 IU, for example) may be needed during one treatment cycle before a response is obtained in the patient. Two recent reports provide comparisons between the levels of plasma FSH and LH during induced and natural cycles (Czygan et al., 1972; Fogel et al., 1972). Treatment with clomiphene is cheap and has the advantage of stimulating the release of endogenous gonadotropins (Bishop, 1970). These treatments have enabled many couples to have their own children. The dangers are ovarian hyperstimulation, which can have such adverse consequences as ovarian enlargement, clotting, and ascites (Mozes et al., 1965), and multiple pregnancies leading to great obstetric risks for mother and fetuses. No studies were made on the actual timing of oocyte maturation and ovulation, and the methods of treatment that were designed for amenorrheic women had to be adapted for women with a normal menstrual cycle.

The routine treatment of these women was introduced by Steptoe and Edwards (1970), based on the earlier work. Most of the patients were infertile because of occlusion of their oviducts, and the necessary clinical treatment demanded the recovery of preovulatory oocytes for fertilization and cleavage in vitro, followed by the intrauterine transfer of the embryos. After preliminary trials with extremely low doses of HMG, a standard procedure was adopted of three injections of HMG on days 3-4, 5-6, and 7-8 of the menstrual cycle, the amount of HMG being 225 or 300 IU per injection. Administration of the ovulatory dose of 5000 IU HCG on day 10 or 11 was timed slightly earlier than the expected natural surge of LH, because control over the preovulatory stages of oocyte maturation had to be maintained. The response of the patients, as judged by the number of enlarging follicles in the ovary and the output of urinary estrogens, was satisfactory. Perhaps the secretion of endogenous LH by the cyclic women enhanced the response to the low levels of HMG used, for the two gonadotropins FSH and LH are known to act synergistically (Crooke, 1970). These methods are almost certainly bound to be modified, perhaps to replace gonadotropins with clomiphene, or with the hypothalamic releasing factors which have recently been synthesized (Schally et al., 1971a,b).

Strict control had to be exercised over the timing of oocyte maturation, in order to collect oocytes just before ovulation. As judged from earlier evidence (Edwards, 1965b), diakinesis would occur 28 hours after the initiation of maturation. Some oocytes collected at this time from patients primed with gonadotropins were found to be in diakinesis (Fig. 1) (Jagiello et al., 1968; Steptoe and Edwards, 1970). Diakinesis is an excellent marker of maturation, for it is characteristic, lasts for only 1-2 hours, and occurs some 6-8 hours before ovulation. The recovery of these oocytes thus showed that fine control was being exercised over oocyte maturation, and the time of their collection was therefore advanced to 32 hours post-HCG in order to obtain oocytes immediately before ovulation. The morphological appearance of the follicles at this



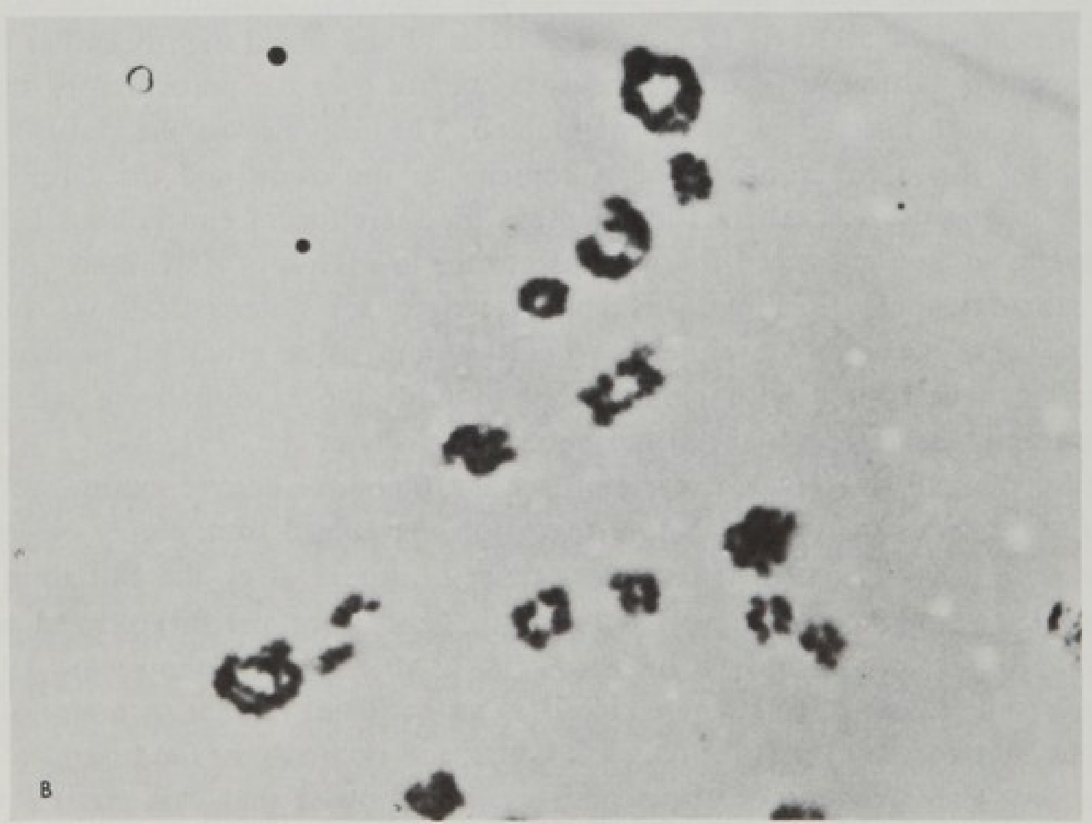


FIG. 1.–(A) Diakinesis in a mouse oocyte, showing one bivalent that is weakly joined or already separated into univalents. (B) Detail from a human oocyte in diakinesis after it was placed in culture for maturation in vitro. (Fig. 1A reproduced by courtesy of the editor of *Nature*.)

time confirmed that ovulation was imminent: large follicles with thinning walls were regularly found, yet no fresh corpora lutea were seen in any of the patients.

Laparoscopic Recovery of Preovulatory Oocytes

Methods of collecting oocytes from Graafian follicles have been developed in the past 2 years. Laparoscopy was the best technique to withdraw the human oocytes, for demands on the patient are minimal, yet various manipulations can be carried out in the body cavity (Steptoe, 1969). The abdomen is distended with an inert gas, e.g., carbon dioxide, to provide a clear view of the organs and space for the surgeon to work. Light from an external source is transmitted via glass fibers into the abdomen, and a slender telescope inserted just below the navel provides the surgeon with clear vision. Manipulating forceps of a special design are then introduced via a minute slit in the abdominal wall to one side of the midline. Initially, the needle of a syringe was passed through the abdominal wall on the opposite side of the abdomen, but later a special aspirator was developed (Fig. 2) (Purdy et al., 1971). Gentle vacuum is used to withdraw the contents of individual Graafian follicles into a collecting dish. Initially, oocytes were obtained from approximately one-third of the follicles aspirated, but improvement in techniques has now led to a recovery rate of 60-70%.

Some oocytes were atretic and possessed few or no cumulus cells. Others were nonovulatory and surrounded with a few layers of corona cells; these could also be identified by the persisting germinal vesicle with its lampbrush-like chromosomes and prominent nucleolus (Steptoe and Edwards, 1970). Most preovulatory oocytes were aspirated from larger follicles, and on average two were collected from each patient. These oocytes are surrounded by the corona radiata, cumulus cells, and often a diffuse clot of "thick follicular fluid" in which a cloud of cumulus cells was embedded.

The rapid identification of preovulatory follicles would assist in assaying the response to hormones, and in studies on fertilization in vitro, by indicating those follicles likely to yield a fertilizable oocyte. One potential method is to assay the levels of steroids in follicular fluids: in the mare, the preovulatory follicle is richer in estradiol-17β than in progesterone (Younglai and Short, 1970). Unfortunately, no correlation has been detected between the levels of estradiol and progesterone and the state of development of the human follicle (Edwards et al., 1972). Analyses of pyruvate levels in the fluid might be valuable, for this intermediary is synthesized during preovulatory development in mouse follicle (Donahue and Stern, 1968).

Maturation of Oocytes in Vitro

The postdictyotene events which normally occur in oocytes after stimulation with endogenous LH can be induced experimentally. When oocytes in dictyotene are released from their Graafian follicles into a suitable culture

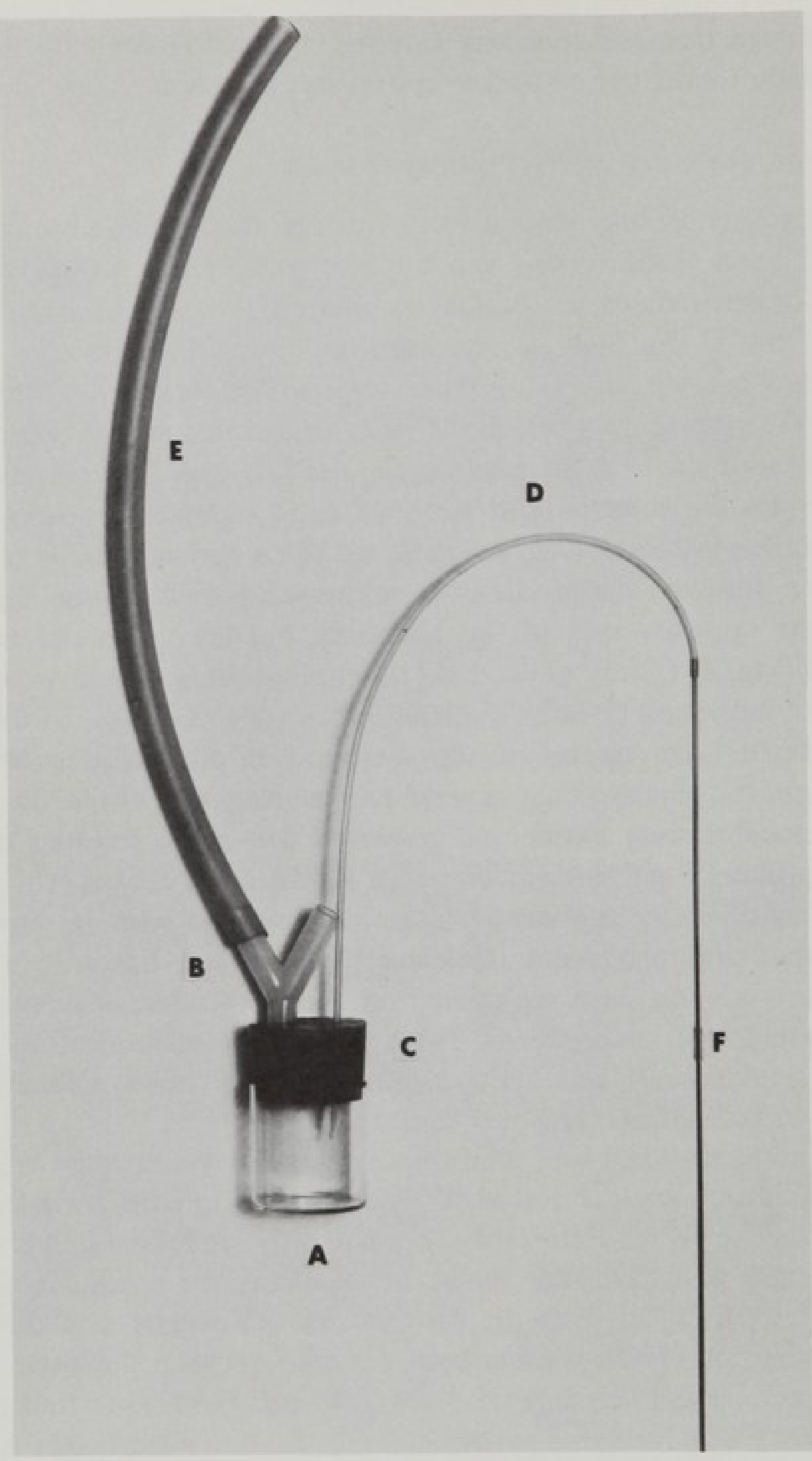


FIG. 2.–Aspirator for withdrawing the contents of follicles. Vacuum through the wide arm (E) is directed through the needle when required by simply blocking the open Y arm (B) in the aspirator. Oocytes and follicular fluid are drawn through the needle (F) and its lead (D) into the collecting chamber (A). The collecting chamber can be quickly removed from the neoprene bung (C) and replaced.

medium, they resume meiosis just as if LH had been given to the female (Pincus and Enzmann, 1935; Chang, 1955a; Edwards, 1965a, 1965b). Various media have been employed and mouse oocytes were recently matured in a defined medium without protein (Haidri et al., 1971).

Human ovarian tissue is often excised for clinical reasons and several oocytes can usually be recovered (Edwards, 1965b). The earliest report on the maturation of human oocytes in vitro appeared more than 30 years ago (Pincus and Saunders, 1939). A precise study of the sequence of events during egg maturation is necessary if specific stages of meiosis or oocytes ready for fertilization are needed. Many studies have provided estimates of the rate of maturation of oocytes in vitro of various mammalian species (Schuetz, 1969; Bibliography of Reproduction, 1970; Chung, 1971). Marked differences occurred in the time required to complete maturation, although for any one species the rate of maturation was similar whether occurring in vitro or in vivo (Chang, 1955a; Edwards, 1965a). Human oocytes matured in vitro in medium 199 supplemented with fetal calf serum remained in dictyotene for at least 24 hours before entering diakinesis (Fig. 1); by 30 hours the majority were in metaphase I and most of them had reached metaphase II by 11 hours later (Table 1) (Edwards, 1965b).

Recent reports also describe the maturation of human oocytes in vitro (Table 1). Almost two-thirds matured in a chemically defined medium containing pyruvate and bovine albumin (Kennedy and Donahue, 1969a). Variations have been reported in the timing of maturation. Estimates made by Yuncken (1968) and by Suzuki and Iizuka (1970) were evidently similar to those published by Edwards (1965b). The use of a medium containing pyruvate (Bavister, 1969) in combination with follicular fluid evidently accelerated maturation to 24 hours or less (Edwards, 1970a). The use of microdrop cultures also resulted in a more rapid maturation (Jacobson et al., 1970), although these results must be questioned since the oocytes were scored through the cumulus cells and there is some confusion about the relationship between metaphase I and the extrusion of the first polar body. Many oocytes were delayed in metaphase I between 46 and 50 hours in culture, as judged by phase contrast or electron microscopy, when incubated in medium F10 and human serum (Mastroianni and Noreiga, 1970). Few oocytes matured, and some showed accelerated maturation in undiluted fetal calf serum (Chandley, 1971). Precise timing of maturation can be made from squash preparations of oocytes, and some of Chandley's preparations appear to be in dictyotene rather than diakinesis at 24 hours. It is evident that the effect of various media and conditions of culture on the maturation of human oocytes requires further study.

How do these estimates of maturation in vitro compare with the maturation of the human oocyte in its follicle? The estimates made by Edwards (1965b) were similar to those observed in vivo following the injection of LH preparations

TABLE 1–*Maturation of Human Oocytes in Vitro*

Culture medium	Total no. of oocytes	Hours in culture					% of oocytes maturing	Reference
		0-24	25-27	28-31	32-36	>36		
199 plus 15% calf serum	131	31 GV, 1 MII	3 GV, 6 Diak, 2 MI	1 GV, 1 Diak, 13 MI	1 GV, 5 MI, 1 Telo-I, 1 MII	16 GV, 3 MI, 46 MII	80	Edwards (1965b)
199 plus 15% fetal calf serum	49					5 MI,* 20 MII	51	Kennedy and Donahue (1969)
F 10 plus bovine serum albumin	38					5 MI,* 19 MII	63	Kennedy and Donahue (1969)
Hank's saline with 20% fetal calf serum and 5% human follicular fluid	33	12 GV, 21 MI		5 GV,† 28 MI inseminated	7 MI,‡ 19 MII, 1 Deg	1 MI, 23 MII, 2 Deg	Approx. 79	Jacobson et al. (1970)§
F 10 plus 10% human serum		3 GV, 1 Deg				19 GV, 18 MI, 23 MII (1st PB), 8 Deg	60	Mastroianni and Noriega (1970)

Calf serum		M II oocytes at 19 hr		30	Chandley (1971)
199 and 0.4% bovine serum albumin	109		36 D-P, 11 MI, 9 MII, 53 Deg	18	Chung (1971)¶
199 plus heated follicular fluid	140		44 D-P, 25 MI, 44 MII, 27 Deg	50	Chung (1971)

GV = germinal vesicle, Diak = diakinesis, MI = metaphase I, Telo-I = telophase of first meiotic division, MII = metaphase II, D-P = prometaphase, Deg = degenerating, 1st PB = first polar body extruded.

* Oocytes scored between 43 and 47 hr.

† Observations at 30 hr.

‡ Observations at 32 hr.

§ Classified as MI by scoring for first polar body.

¶ Incubated for 48 hr.

into patients (Jagiello et al., 1968; Steptoe and Edwards, 1970). In both of these studies, some oocytes found in metaphase II at 28-30 hours could have been atretic, a situation known to occur in atretic follicles in vivo. Alternatively, there may be slight differences in the rate of maturation of different oocytes.

The ultrastructural characteristics of human oocytes maturing in vitro are similar to those maturing in vivo, except for degenerative changes in some of them (Zamboni, 1971). By contrast, similar forms of degeneration were found in oocytes of *M. mulatta* matured in vitro and in vivo (Thompson et al., 1971).

FERTILIZATION

Fertilization takes place in the ampulla of the oviduct in most species, and the preliminary events of sperm transport through the female tract are well known (Austin and Bishop, 1957a, 1957b). In the human female, few spermatozoa reach the ampulla; these evidently remain active for 2-3 days. The ovulated ova are enveloped by cumulus and corona radiata cells, which may be dispersed by hyaluronidase and other enzymes liberated from spermatozoa in most species. Spermatozoa must undergo a change known as "capacitation" before they can penetrate into the ova (Austin, 1951; Chang, 1951), as shown for example by the delay between the arrival of spermatozoa at the site of fertilization and their entry into the eggs (Austin and Braden, 1954; Chang, 1955c; Adams and Chang, 1962; and others). Ova can be fertilized several hours after ovulation.

The second meiotic division in the oocyte is resumed as the spermatozoon enters the egg. Anaphase and telophase are completed rapidly, the second polar body is extruded, and two pronuclei are formed (Austin, 1961). The fine structure of a human pronucleate ovum has been described by Zamboni et al. (1966). The two pronuclei grow and move toward each other, but never fuse; at syngamy, they each enter prophase simultaneously, and the maternal and paternal chromosomes become aligned on the cleavage spindle. Syngamy is often considered as the final stage of fertilization.

Oocytes for studies on fertilization are usually obtained by one of three methods. Oviducal oocytes can be collected after ovulation during the natural estrous or menstrual cycle. Second, the injection of gonadotropins induces the ovulation of large numbers of eggs at a precise time. Last, oocytes that have completed all or part of their maturation in vitro can be used for studies on fertilization. Oocytes matured in vitro are very convenient for studies on human fertilization, but have disadvantages as compared with those obtained from the oviducts after ovulation. Mouse or rabbit oocytes fertilized in vivo following their maturation in vitro seldom developed into viable blastocysts or fetuses (Chang, 1955b; Cross and Brinster, 1970; Edwards, 1970b). Pig oocytes matured in culture displayed abnormal forms of fertilization (data of Polge and Edwards,

mentioned in Edwards, 1966). A few of the human oocytes matured and fertilized in vitro displayed cytoplasmic fragmentation (Bavister, et al., 1969; Edwards, et al., 1969). If oocyte maturation begins in the follicle and is completed in vitro or in the oviduct, normal midterm fetuses can be obtained in various species (Chang, 1955b; Cross and Brinster, 1970; Edwards, 1970b; Leman and Dzuik, 1971). Until methods can be developed for maturing oocytes more successfully, they must be collected just before or after ovulation if fertilization and embryonic development are desired.

Fertilization of Mammalian Eggs in Vitro

The fertilization of mammalian eggs in vitro was initially difficult to accomplish. Leads had to come from the analysis of fertilization in vivo, and recurrent discussion has concerned the need to obtain capacitated spermatozoa from the uterus of a mated female for studies on fertilization in vitro. Capacitation in vivo requires 6 hours in the rabbit, but less time in mice, rats, and hamsters; details can be found in several recent symposia (Chang, 1968, 1969; Austin, 1969a; Brackett, 1969a, 1971; Bedford, 1970).

The stimulus to capacitation and the consequent biochemical changes are still poorly understood. On the other hand, the ultrastructural changes occurring in the gametes at fertilization are now known in some detail. The outer acrosomal membrane and plasma membrane fuse and vesiculate as the spermatozoa pass between the cumulus cells, changes that probably facilitate the release of hyaluronidase and other enzymes (Bedford, 1969). Different enzymes are evidently released from the spermatozoon for its passage through the zona pellucida. Debate exists about the nature of these "zona lysins" and their location in spermatozoa (Bedford, 1969, 1970; Yanagimachi and Noda, 1970). The plasma membrane in the postnuclear cap of the spermatozoon fuses with the plasma membrane of the ovum to effect the entry of the spermatozoon. Thus the principles of fertilization are becoming clear, except for the initial steps in capacitation. Various theories have been proposed to account for the initiation of capacitation; we suggest that progesterone in the follicular fluid or from the cumulus cells is probably involved in vesiculation because acrosomes are modified lysosomes (Allison and Hartree, 1970), and progesterone destabilizes lysosomal membranes (Weissman, 1969). The cumulus cells are necessary for the capacitation of mouse epidydimal spermatozoa, although fertilization occurs with uterine spermatozoa in the absence of cumulus cells (Pavlok and McLaren, 1972). Various substances in seminal plasma or on the plasma membrane could exert an inhibiting effect on fertilization (e.g., Williams, et al., 1969).

Progress with the fertilization of mammalian eggs in vitro has been rapid during the past few years. Early observations on uterine capacitation led Moricard (1954), Dauzier and Thibault (1956), and Chang (1959) to use uterine

spermatozoa for fertilizing rabbit eggs in vitro. Subsequently, uterine spermatozoa were employed widely in rabbits and other species for fertilization in vitro, e.g., in mice (Whittingham, 1968). The shift away from uterine spermatozoa began when Yanagimachi and Chang (1964) fertilized hamster eggs in vitro using epididymal spermatozoa suspended in a Tyrode's solution (see also Barros and Austin, 1967). Uterine spermatozoa were reported to be more effective than epididymal spermatozoa (Yanagimachi, 1966), although this view was not confirmed by others (Hunter, 1969). Later, the capacitation of hamster spermatozoa was induced by follicular fluid (Yanagimachi, 1969a, 1969b).

The conditions required for fertilization in vitro were ill defined; even the pH of the medium was not standardized until Bavister (1969) finally achieved consistent rates of fertilization in hamster eggs using a pH controlled at 7.6. Brackett and Williams (1968) used a similar pH to fertilize rabbit eggs in vitro, although they were then using uterine spermatozoa (Brackett, 1971). A calcium concentration of 0.02% was most favorable for the penetration of mouse eggs in vitro (Iwamatsu and Chang, 1971). Bavister's methods have now proved suitable for fertilizing human eggs in vitro (Edwards et al., 1969; Bavister et al., 1969), a few cow eggs (Edwards, unpublished observation), and mouse eggs (Austin et al., 1971), and to obtain penetration of spermatozoa through the zona pellucida of rabbit eggs (Austin et al., 1971). The interval between insemination and penetration of mouse eggs was reduced to 20 min or less by previous exposure of the spermatozoa to bovine follicular fluid (Iwamatsu and Chang, 1970). Recently, however, more data have shown that follicular fluid is dispensable, for Toyoda et al. (1971a, 1971b) showed that capacitation occurred in a simple defined culture medium without exposing mouse spermatozoa to any part of the female tract. Penetration began immediately after the eggs were placed in the sperm suspension (Table 2). This is obviously almost the final step in controlling fertilization in vitro.

Bavister's medium was slightly modified for studies on the fertilization of human eggs in vitro (Table 3), so that the ionic components approximated to levels found in human follicular fluid. Oocytes matured in vitro or removed from their follicles by laparoscopy were transferred through two changes of Bavister's medium and placed in a suspension of spermatozoa that had been washed by light centrifugation. The timings of fertilization are given in Table 4, and illustrations are shown in Fig. 3. Most of the fertilized eggs were bipronucleate, although one was found to have three. Some anomalies of fertilization were seen in oocytes previously matured in vitro, such as the failure of spermatozoa to pass completely through the zona pellucida and fragmentation of pronuclei. Bavister's medium could be replaced by medium 199, Waymouth's medium MB 752/1, or other media provided they were adjusted to a high pH and pyruvate was added.

Earlier studies on the fertilization of human ova were reported by Menkin

TABLE 2–*Effect of Sperm Preincubation Time on the Fertilization Rate One Hour After Insemination**†

Sperm preincubation time (min.)	No. eggs examined	No. (%) eggs penetrated	No. (%) eggs fertilized
Control, no preincubation	101	23 (22.8)	11 (10.9)
3-7	158	38 (24.1)	9 (5.7)
15	107	75 (70.1)	50 (46.7)
30	114	107 (93.9)	84 (73.7)
60	126	126 (100.0)	121 (96.0)
Control, no preincubation	115‡	108 (93.9)	102 (88.7)

* From Toyoda et al. (1971a, 1971b).

† Sperm recovered from cauda epididymis were incubated in a chemically defined medium.

‡ Examined 3 hr after insemination.

and Rock (1948), Shettles (1955), and Hayashi (1963), and each reported that an occasional ovum cleaved after the admixture of oocytes and spermatozoa. In these studies, however, the midpiece was not identified in any ovum, and the detection of this structure must be considered at present as the best criterion to judge whether penetration has occurred. In our own earlier studies human fertilization was not achieved using various media, spermatozoa from the cervical mucus, or an animal oviduct (Edwards et al., 1966). In a recent study, Jacobson

TABLE 3–*Medium for Human Fertilization in Vitro**

	Mg/liter
NaCl	7500
KCl	390
$NaH_2PO_4 \cdot 2H_2O$	57
$NaHCO_3$	3295
D-Glucose	1000
$CaCl_2$	200
$MgCl_2 \cdot 6H_2O$	100
BSA	3600
Phenol red	17
Na pyruvate	11
Penicillin	100 IU/ml
Medium then buffered to approximately pH 7.55 with bicarbonate	

* Formula modified from Bavister, (1969).

TABLE 4–*Timing of the Fertilization of Human Eggs in Vitro**

Time after insemination (hr)	Details of events				
	Unpenetrated	Sperm in zona pellucida	Sperm in perivitelline space	Sperm in vitellus	Pronucleate
6-6½	3				
7-10½	5	2	4		
11½ and later	9	5	1	1	11†

* From Bavister et al. (1969).

† More than 38 other ova have been seen to be pronucleate by low-power microscopy in later work (Edwards et al., 1970).

et al. (1970) did not examine eggs for a midpiece, but claimed to identify a Y chromosome during syngamy. Identification of the Y chromosome in cleaving embryos would obviously prove beyond doubt that fertilization had occurred, but Jacobson et al. do not produce a karyotypic analysis. Definite identification of the Y would be provided by fluorescence methods (Caspersson et al., 1970; Pearson et al., 1970), but our own studies (with P. L. Pearson and M. Bobrow) have shown that fluorescence of all structures is greatly reduced in the nuclei of human preimplantation embryos.

In another recent study Seitz et al. (1971) used human oocytes matured in vitro for fertilization, but produced no evidence that oocyte maturation was normal other than the presence of extruded bodies considered to be polar bodies. Maturation in vitro should be tested by more rigorous methods such as the identification of diakinesis in a few oocytes. They also did not look for midpieces, but relied on cleavage of the eggs as evidence of fertilization; they found that fertilization rarely occurred unless human spermatozoa were first incubated in the uterus of a monkey. This observation takes us back to uterine capacitation. Seitz et al. used Ham's F10 for fertilization; in our studies (Edwards et al., 1970) this medium seemed unsuitable for fertilization because it seemed to encourage overgrowth of the cumulus cells.

Parthenogenetic Activation of Mammalian Ova

Ova of many species including man will undergo a form of rudimentary parthenogenic development if they are not fertilized several hours after ovulation. Parthenogenesis has been induced experimentally in eggs of various species, e.g., by chilling or heating activated rabbit ova, and claims were made that viable offspring were born (Pincus, 1939), but the experiment has been criticized since accidental fertilization could not be excluded (Beatty, 1957).

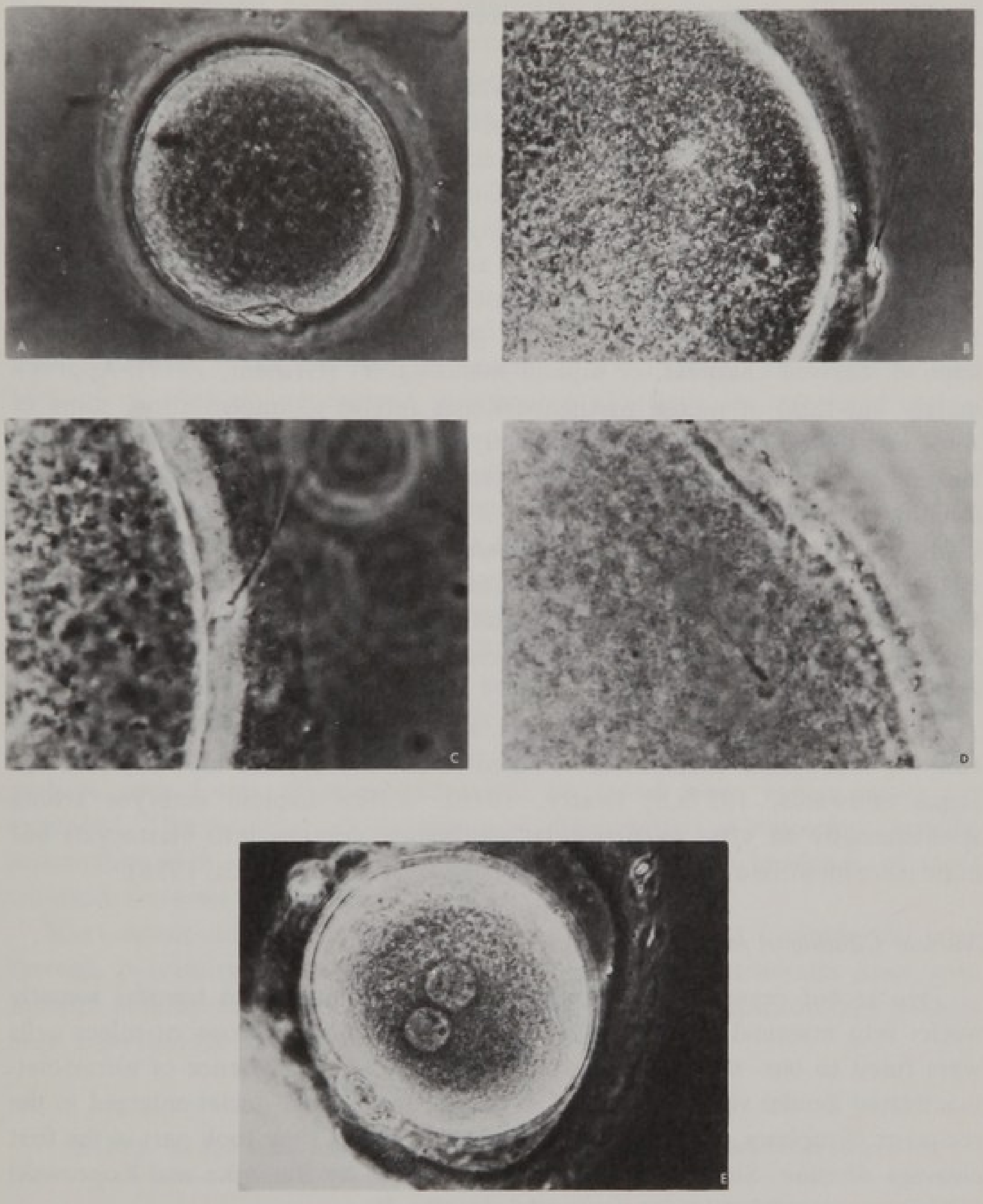


FIG. 3.–Successive sequences in human fertilization in vitro. (A) Unfertilized living egg with first polar body. (B) Spermatozoon penetrating through the zona pellucida of a living egg. (C) Sperm head just touching the vitellus of a living egg. (D) Swelling sperm head and its midpiece in a fertilized egg that has been fixed and stained. (E) Two pronuclei and polar bodies in a fertilized living ovum. (Figs. 3B and C by courtesy of the editor of *Nature* Figs. 3D and E by courtesy of the editor of the *Journal of Reproduction and Fertility.*)

Parthenogenetic rabbit blastocysts were obtained by chilling rabbit ova (Thibault, 1947; Chang, 1954).

A mild electrical stimulus to the oviduct resulted in the activation of the mouse egg and development beyond implantation (Tarkowski et al., 1970). Brief exposure to hyaluronidase in vitro also activated mouse ova, and various forms of pronuclear behavior were observed (Graham, 1970, 1971). Many of the eggs were considered to be diploids retaining some heterozygosity, since they contained chromosomes of the oocyte and second polar body; in others with two pronuclei, a common metaphase plate was not formed at syngamy, and each nucleus divided independently. Mouse parthenogenones thus appeared to be haploid, haploid chimeras, or diploid homozygotes (Graham, 1971). Approximately one-third of mouse parthenogenones survive to implantation, some of these being presumed diploid (Tarkowski, 1971). A head process, heart, somites, and limb buds were found by midgestation in some implants, these being diploid. By the ninth or tenth day only 2 out of 90 implantation sites contained living embryos (Tarkowski, 1971). Many parthenogenones could have been viable because they retained some heterozygosity through the incorporation of the second polar body into the egg, although this explanation seems not to explain the death of those parthenogenones derived from inbred lines (Tarkowski, 1971; Graham, 1971).

Curiously, gynogenetic embryos (i.e., a normal egg fertilized by spermatozoa carrying inactivated chromosomes) failed to develop beyond the early cleavage stages (Edwards, 1957a,b; Beatty, 1970). A few haploid embryos arising spontaneously or after experimental treatments develop into blastocysts but none were identified after implantation (Beatty, 1957; Edwards, 1958).

Nuclear Cloning of Mammalian Eggs

Two recent reports have appeared describing attempts to transfer somatic nuclei into mammalian ova. Embryonic and adult bone marrow or spleen cells were fused to one- or two-celled mouse embryos in the presence of ultraviolet-inactivated Sendai virus (Graham, 1969). The transferred nuclei enlarged in the recipient cytoplasm, and some evidence indicated that they took part in the first cleavage division. Similar observations were made by Baranska and Koprowski (1970), some ova developing into early morulae.

Virus Infection of Oocytes and Fertilized Eggs

The zona pellucida is thought to protect the mammalian embryo from infection by large viruses although small viruses such as Mengo encephalitis virus can penetrate through this membrane (Gwatkin, 1964, 1966). Two reports have shown that unfertilized and clearing mammalian ova can be infected by SV40 or MSV viruses after removal of the zona pellucida, although their development to

blastocysts appeared to be largely unaffected (Baranska et al., 1971; Sawicki et al., 1971). A virus (SV40 DNA) infecting rabbit spermatozoa can be carried into the ovum during fertilization (Brackett et al., 1971). No genetic effects during embryonic or fetal development arising from virus infections of ova have been reported.

CHROMOSOMAL ANOMALIES IN OOCYTES AND PREIMPLANTATION EMBRYOS

Many human abortuses or neonates are chromosomally imbalanced (Carr, 1965, 1970a; Dhadial et al., 1970; and many others), although there are fairly wide variations in the reported frequencies (Carr, 1970a). Carr estimated that 36% of all clinically recognized spontaneous abortions are associated with a chromosomal anomaly. This figure does not include preimplantation and early postimplantation losses, which are difficult to assess. Almost one-half of spontaneous abortions during the first trimester were aneuploid, reducing to 15% in the second trimester (Dhadial et al., 1970). In this series, 46% of the imbalanced fetuses were autosomal trisomics, 30% were sex-chromosome monosomics (45X), and 13% were triploids. The incidence of aneuploids was equally high in the series described by Arakaki and Waxman (1970). Trisomy for each chromosome group has now been identified in spontaneous human abortuses; the incidence is highest for group E and lowest for groups A, B, and F (Table 5). There can be little doubt that still more imbalanced fetuses, and perhaps new forms of aneuploidy, will be found when earlier stages are examined. The incidence of chromosomal abnormalities at conception may be remarkably high, especially in older mothers, since the incidence of most trisomics increases with maternal age (Carr, 1965).

What causes this chromosomal imbalance in man? Most triploids will arise through polyandry or polygyny at fertilization. Uniform trisomics must arise through nondisjunction during meiosis or an early cleavage division, and mosaics must arise through an error in later cleavage.

TABLE 5–*The Incidence of Various Trisomics in Human Spontaneous Abortions*

	No. of abortuses classified according to their trisomic chromosome group							
Reference	A	B	C	D	E	F	G	Total
Dhadial et al. (1970)	–	–	6	11	12	–	10	39
Carr (1971c)	1	2	3	11	15	3	8	43
Lazar et al. (1971)	8	6	51	44	83	4	49	245
Combined data	9	8	60	66	110	7	67	327

The Origin of Chromosomal Anomalies During Meiosis

The control of oocyte maturation in vitro or in vivo has opened opportunities for studying the meiotic chromosomes of oocytes. Somewhat disappointingly, preparations of human chromosomes have not been of high quality, and most studies have been carried out on mouse oocytes. Chiasma frequency declined with increasing age of the mother, from 25 to 21 per oocyte between 3 and 12 months of age in CBA females, and slightly less steeply in C57BL females (Henderson and Edwards, 1968). Similar observations have been made on six inbred mouse strains (Jagiello and Polani, personal communication). Chiasma frequency in oocytes was lower than in spermatocytes. The reduced chiasma frequency in oocytes of older mothers was associated with a localization of the chiasmata to the terminal ends of the chromosomes, and with the presence of univalents in some oocytes (Henderson and Edwards, 1968). An abrupt decline in chiasma frequency between diakinesis and metaphase I might be due to terminalization of chiasmata during this brief period (Ford, personal communication).

According to Edwards and Henderson (1970), human oocytes displayed similar characteristics. Chiasma frequency in 15 oocytes was estimated to be between 37 and 48, and univalents were detected (Edwards and Henderson, 1970). The numbers of chiasmata were lower than those reported by Kjessler (1966, 1970) in human spermatocytes. Fifty-two chiasmata were counted in a human oocyte (Yuncken, 1968), although later results gave between 36 and 52 chiasmata per oocyte (J. H. Edwards, 1972). Estimates by Henderson of the chiasmata in oocytes matured in vivo (Jagiello et al., 1968) were between 46 and 51. A human oocyte examined by Chandley (1971) had not more than 40 chiasmata.

The decline in chiasma frequency in aging mothers could arise in two ways. Chiasmata might be lost through terminalization during the long dictyotene stage, or fewer chiasmata might have been formed in oocytes of older females. One method of deciding between these alternatives is to analyze the recombination frequency of linked genes in the offspring of aging mothers. Terminalization would not alter recombination frequencies, since it occurs after the chiasmata have formed. On the other hand, a decline in the number of chiasmata formed at zygotene in some oocytes would be reflected in a decline in recombination frequency.

Data on variations in recombination frequency in offspring of aging mothers are not extensive. In mice, fewer recombinants were found between the linked genes Agouti/undulated (Fisher, 1949), pallid/fidget (Bodmer, 1961), and leaden/fuzzy (Reid and Parsons, 1963). In one of these studies, recombination frequency increased with paternal age (Reid and Parsons, 1963). Data on recombinations between each of eight pairs of closely linked genes in man were pooled, because so little evidence was available for each linkage separately, and a

significant excess occurred in the last half of the sibships (Svejgaard et al., 1971; Weitkamp, 1971). This evidence suggests that more recombinants occurred in the gametes of older parents, both male and female. In contrast, data on loosely linked loci, especially for the linkage AK, nail-patella/ABO, and Rh/6-PGD, revealed a significant decline in the number of recombinants in older mothers, a conclusion in accord with the data from mice (Weitkamp, 1971). Most of the available data thus suggest that fewer recombinants occur in offspring of older females, which implies in turn that the decline in chiasma frequency in older oocytes is due to the formation of fewer chiasmata in them at zygotene. If these conclusions are valid, oocytes with fewer chiasmata must be selectively conserved until later life (Henderson and Edwards, 1968). Gradients during oocyte formation in the fetal ovary could result in fewer chiasmata in some oocytes. Physiological conditions in the fetal ovary must alter during the period of oocyte formation, as shown by the waves of atresia at different embryological stages (Baker and Franchi, 1967b). In plants and insects, developmental, nutritional, or experimental treatments are known to inhibit chiasma formation (Rees and Naylor, 1960; Henderson, 1970).

If fewer chiasmata and more univalents exist in oocytes of older mothers, the relationship between trisomy and maternal age would be explained. Unfortunately, the observations on univalents have not been confirmed. Terminal chiasmata, but no univalents, were found in human oocytes (Uebele-Kallhardt and Knörr, 1971). Likewise, chiasmata were found at the terminal ends of mouse chromosomes in the oocytes of older mothers, but no univalents were found during diakinesis and metaphase I (Jagiello and Polani, personal communication). Moreover, these workers also discovered that all mouse and human oocytes examined at metaphase II were diploid. This evidence would exclude anomalies arising during the first meiotic division, and up to metaphase of the second meiotic division, as causative factors in trisomy.

Nondisjunction could occur during anaphase II, i.e., after fertilization, especially under conditions leading to disruption of the spindle. Delayed fertilization could precipitate nondisjunction, for the spindle becomes distorted and displaced and chromosomes scatter in the cytoplasm of the egg (Charlton, 1917; Edwards, 1957a; Austin, 1967; Szollosi, 1971). Delayed fertilization in older women resulting from the reduced frequency of intercourse in older couples has been postulated as a cause of trisomy in man (German, 1968), although statistical analysis failed to support this suggestion (Cannings and Cannings, 1968). The premature separation of chromatids found in mouse oocytes several hours after ovulation could result in trisomic embryos (Rodman, 1971). Chromatid separation at metaphase II must occur selectively with particular chromosomes if it causes human trisomy, otherwise trisomics of all chromosomes would occur as frequently as those for groups E and G, and many polysomics would be expected. Selective nondisjunction has been observed in

the mitoses of cultured human cells, but reports on the patterns of chromosome loss are conflicting (Neurath et al., 1970; Zang and Zankl, 1970). Perhaps the most effective argument against nondisjunction in anaphase II as a cause of trisomy comes from animals where delayed ovulation or delayed fertilization was deliberately invoked. Delayed ovulation in rats resulted in 18 out of 390 embyos being chromosomally unbalanced, most of them mosaics, as compared with 6 out of 410 in controls (Butcher and Fugo, 1967). Likewise, delayed fertilization of rabbit and mouse eggs did not lead to trisomy in the embryo (Austin, 1967; Shaver and Carr, 1967; Vickers, 1969b), and most anomalies were due to triploidy or mosaicism. Conclusions about nondisjunction at anaphase II thus remain speculative at present.

We have reached the conclusion that nondisjunction at anaphase I or anaphase II has not been proved as a cause of trisomy in man. Clearly, more critical experiments are required; there are obvious drawbacks in extrapolating to man from animals where there is a low frequency of trisomic fetuses. Methods such as maturation in vitro could give misleading information about events occurring in the intact ovary. There is clear evidence that nondisjunction of the sex chromosomes does occur in the testis, and in either oocyte or embryo, from studies on the sex-linked gene Xga (Race and Sanger, 1969); the extra X was shown to be maternal in 60% of XXY's and the paternal X had been lost in 74% of XO's. Some family evidence provided direct proof that XXY's could arise from nondisjunction during the first or second meiotic division in the testis.

Other explanations of the origin of human trisomics have little supporting evidence. A slower dispersal of nucleoli in older oocytes was postulated to interfere with chiasma formation (Polani et al., 1960), but chiasma formation occurs before dictyotene. A nucleolus persisting into metaphase was advocated by Evans (1967) because the X and groups D, E, and G, which are most commonly involved in trisomy, have associated nucleoli. External factors can influence the incidence of trisomy or other chromosomal anomalies. The incidence of chromosomal aberrations is influenced by geographical distribution (Stevenson et al., 1966; Robinson and Puck, 1967; Ross et al., 1967) and season of the year (Robinson and Puck, 1967; Jongbloet, 1970). Statistical evidence indicated that infections could be a source of trisomy (Stoller and Collman, 1965a,b), and chromosome breaks have been found in cells that have been infected with viruses (Huang, 1967; Kato, 1967). A correlation between the trisomy 21 syndrome and viral hepatitis is frequently indicated (Stoller and Collman, 1965a, 1965b) and as frequently denied (Leck, 1966; Stark and Fraumeni, 1966). Adenovirus causes nonrandom damage to human fetal cells in vitro (McDougall, 1970). Aneuploidy has been observed in meiotic chromosomes of the testis in mouse stains with latent ectromelia (Schroder et al., 1970). Treatment of male mice with LSD increased the incidence of breaks, gaps, and fragments (Cohen and Mukherjee, 1968; Skakkeback et al., 1968).

Steroid hormones are reported to exert structural effects on meiotic chromosomes, which became adhesive in canine testes perfused with progesterone (Williams et al., 1968). Triploid and XO fetuses, in addition to other anomalies, were found in women who became pregnant after discontinuing oral contraception (Carr, 1967, 1970b), an observation given some support by Dhadial et al. (1970). However, selected estrogens and progestins had no influence on human chromosomes in vitro (Stenchever et al., 1969). This is not surprising, since high concentrations of steroids were found in human follicular fluids sampled just before ovulation was due (Abraham et al., 1970, and unpublished observation).

Anomalies at Fertilization and Chromosomal Disorders

Information on human fertilization is still scanty, but a wealth of knowledge has accumulated on the genetic consequences of anomalous forms of fertilization in animals (e.g., Austin, 1969b,c; Beatty, 1970; Carr, 1971a; Thibault, 1971). We will therefore deal very briefly with this aspect of conception. Nondisjunction during anaphase II has been described above and will not be considered here.

The dispermic fertilization of an egg will lead to triploidy (diandry). Retention of the first or second polar body, usually the latter, will also lead to triploidy (digyny). Triploidy is relatively common in man. Approximately 5-17% of spontaneous human abortions are triploid (World Health Organization, 1966; Dhadial et al., 1970; Carr, 1971b). Triploids rarely survive to full term, although occasional reports of uniform triploid neonates, including prematures, have appeared (J. H. Edwards et al., 1967; Keutel et al., 1970; Schindler and Mikamo, 1970).

In animals, delayed fertilization greatly increases the incidence of diandry and digyny, although there are species variations. Diandry is more common in rats and pigs (Austin and Braden, 1953; Odor and Blandau, 1956; Thibault, 1967); as many as 21% of pig zygotes might be triploid after delayed fertilization (Thibault, 1959). In rabbits, the increase in triploidy after delayed fertilization (Austin, 1967; Shaver and Carr, 1967) is evidently due to digyny, for none of 10 triploid blastocysts had an XYY constitution (Shaver and Carr, 1969). In contrast, delayed fertilization of mouse eggs rarely results in triploidy (Gates and Beatty, 1954; Vickers, 1969b). In rats, mosaic or aneuploid embryos were the most common anomaly after ovulation was artificially delayed (Butcher and Fugo, 1967). There could well be other causes of triploidy; for example, giant oocytes in rodents could be diploid (Austin, 1969c).

The wide species differences in response to delayed fertilization make extrapolations to man of doubtful value. In 31 human triploids, including abortuses, the ratio was 2XYY:20XXY:9XXX (Polani, 1969). Another study

reported a ratio of 1XXX:2XXY and very few XYY (Schindler and Mikamo, 1970). Obviously the XYY's and presumably some of the others arose through diandry. One human egg with three pronuclei was found to possess two sperm midpieces, indicating that it was dispermic (Edwards et al., 1969). Schindler and Mikamo believe that overripeness of the egg favors dispermy rather than digyny; should XYY triploids be less viable, then the excess of XXY triploids may be explained by the predominance of diandric triploids. An increased incidence of triploid abortuses was found among women conceiving soon after discontinuing oral contraceptives, although the cause of triploidy remains obscure (Carr, 1967, 1970b; Haller, 1971).

Tetraploidy is not as common as triploidy (Boué and Boué, 1970) and is lethal in man, although tetraploid cells have been recovered from human abortuses (Carr, 1971b). None of the seven tetraploid specimens described by Carr contained an embryo, All tetraploid abortuses so far recovered have been XXYY or XXXX indicating their likely origin through a failure of cytokinesis at the first cleavage division. Various other defects of fertilization have been reported in animals. The use of exogenous gonadotropins induces the defective development of the zona pellucida or its complete absence from some baboon ova (Katzberg and Hendrickx, 1966), and degeneration of ova in monkeys (Roussel et al., 1969), although excess HCG does not induce aneuploidy in developing rabbit ova (Shaver, 1970). Experimental treatment on spermatozoa and oocytes, such as the use of X-rays, antimitotic agents, etc., also induce chromosomal or other alterations in ova, the experiments being too numerous to mention here (reviewed by Beatty, 1957, 1970). One of the most interesting observations has been the elimination of an X chromosome in pronucleate mouse eggs by X-irradiation, the embryos developing as XO's (Russell and Saylors, 1960; Russell, 1961). Mouse embryos presumed to be YO die in the two-celled stage (Morris, 1968), although many embryos with nondiploid chromosome complements grow into blastocysts (Beatty, 1957, 1970). A wide variety of aneuploid meiotic figures and chromosomally imbalanced blastocysts has been identified in hybrids between the house mouse and the tobacco mouse (Tettenborn and Gropp, 1970; Gropp, 1971).

Spontaneous Mosaic and Chimeric Embryos

Mosaic embryos are defined as having two or more cell lines arising in the zygote itself, e.g., through nondisjunction or chromosome lagging. All female offspring are thus mosaics, since according to the Lyon hypothesis there are two cell lines each with one active X. Chimeras are defined as embryos with two or more cell lines arising from two separate acts of fertilization, e.g., through the fertilization of an egg and polar body, the fusion of two embryos, the transmission of cells from one twin into another, etc.

Approximately 7% of spontaneous abortions in man are chromosomal mosaics (Polani, 1969). Many of these are sex chromosome mosaics (Ford, 1969). Many patients with Turner's syndrome are mosaics for an XO cell line and a line carrying a normal or structurally abnormal X; a single event might have caused the X monosomy and the structural alteration (Ford, 1969). Human autosomal mosaics are much rarer, although Arakaki and Waxman (1970) found equal numbers of autosomal and sex chromosome mosaics in spontaneous abortions of under 20 weeks' gestation. A few patients (less than 4%) with the trisomy 21 syndrome are mosaics, the degree of mosaicism changing with age in some of them (Taylor, 1968, 1970).

There is no direct evidence from human embryos or primate embryos on the causes or incidence of mosaicism. Delayed fertilization in rabbits was reported to lead to a high incidence of mosaicism (Austin, 1967), although this was not found in other studies (Shaver and Carr, 1967, 1969). In rats, delayed ovulation increased the incidence of mosaicism to 2.6% as compared with 0.7% in controls (Butcher and Fugo, 1967). Rare forms of mosaicism were identified in mouse embryos in their preimplantation stages: one was a mosaic for an isochromosome, and another for a minute chromosome (Vickers, 1969b). Isochromosomes have been found in man (Polani, 1969).

The incidence of human chimeras is difficult to ascertain, since they are detected only in favorable circumstances. A critical review of the known animal and human chimeras has been published by Benirschke (1970). The mode of origin of a chimera will obviously determine its genotype (Ford, 1969). One cause of chimerism could arise at fertilization, for some eggs cleave into two equal cells instead of extruding a polar body. In animals, this phenomenon occurs mostly during the second meiotic division just after fertilization and is perhaps due to the displacement of the anaphase-II spindle to the center of the egg. Thus the spindle of many unfertilized mouse eggs is found in the center of the egg between 14 and 20 hours after ovulation (Szollosi, 1971). Experimental treatments can induce the equal division of animal eggs (Braden and Austin, 1954; Austin, 1969c; Edwards and Fowler, 1970). Such eggs have also been found after natural ovulation in mice; Marston (personal communication) recorded up to 20% of the total number ovulated in some mice, and 5% of them had been fertilized in both halves. This phenomenon has not been seen in more than 70 human eggs fertilized in vitro (Bavister et al., 1969; Edwards et al., 1969, 1970). Double fertilization was involved in the 6 cases in which whole-body chimerism in man can be reasonably established (Benirschke, 1970). Double fertilization of two equal halves of an egg seems more likely to lead to chimerism than the fertilization of an egg and its polar body, for polar bodies are small and soon degenerate. However, an XX/XY human chimera was presumed to arise through double fertilization of the oocyte and its polar body, for it could be established that two sets of maternal chromosomes were present

(Zuelzer et al., 1964). Diverse maternal traits were found in other human chimeras which probably arose through double fertilization (Gartler et al., 1962; Myhre et al., 1965; Lejeune et al., 1967).

Other anomalies that could result in chimeras include two binuclear human oocytes possibly arising by egg fusion in multiovular follicles (Kennedy and Donahue, 1969b). Binuclear oocytes are also found in other species, e.g., mice (Donahue, 1970). The dispermic fertilization of a binuclear oocyte is often quoted as a potential source of chimeras (e.g., Dunn et al., 1968), but such oocytes are rare. Moreover, syngamy usually proceeds normally in multipronucleate eggs (Austin, 1961, 1969b; Edwards, 1961); hence dispermic fertilization of binucleate eggs would yield tetraploids rather than chimeras.

Chimeras could also arise through embryo fusion, although this must be rare in man where usually only one egg is ovulated. Nevertheless, multiovular follicles have been found in women after stimulation with gonadotropins (Jones, 1968), and twin ovulations do occur. Embryo fusion is a distinct possibility in the formation of human chimeras especailly since the cases described by Myhre et al. (1965) and Lejeune et al. (1967) were associated with twinning. A uterine factor arising at implantation in mice has been postulated to induce occasional twinning or fusion of morulae or blastocysts (Mintz, 1971a); intersexes in mice (Hollander et al., 1956) might have arisen in this way (Mintz, personal communication). Mouse embryos are often found tightly attached to each other by the zona pellucida (Edwards and Fowler, 1970), and their implantation while still joined, or their fusion after loss of the zona pellucida, could lead to the formation of chimeras.

METABOLISM OF PREIMPLANTATION EMBRYOS

After fertilization has occurred in the ampulla, embryos cleave successively as they move through the oviduct. Transit through the oviduct, which takes between 3 and 5 days in various mammals, has been estimated as 3-4 days in man (Hertig and Rock, 1945; Hertig et al., 1956; Croxatto et al., 1972). Embryos of various species enter the uterus in different stages of development, e.g., four celled in the pig, morula in the mouse, and probably 8-16 celled in man.

Successive cell divisions in the embryo occur every 12-18 hours, each cleavage having a complete cell cycle with distinct G1, S and G2 phases (Gamow and Prescott, 1970). Mammalian embryos seem to differ from those of lower orders in this respect. The large amount of material in the mammalian oocyte, especially certain enzymes, dominates the metabolism of the embryo even though transcription may begin in the one-celled stage. Maternal and embryonic characteristics are thus present during preimplantation development, and their relative importance for the embryo must be determined.

Nutritional and Cultural Requirements of Mammalian Embryos

Many papers and reviews are available on the preparation of defined media for the culture of mouse embryos (Brinster, 1965d, 1971a; Whitten and Biggers 1968; Biggers, 1971; Whitten, 1971; Whittingham, 1971a). Mouse and rabbit oocytes fertilized in vitro and cultured through cleavage and differentiation into blastocysts will develop to full term when transplanted into recipient females (e.g., Mukherjee and Cohen 1970) (see Table 6).

Cleaving embryos of many laboratory species have been cultured in vitro for various periods of time and then transferred into recipients for further development. The data are too numerous to mention here; some reviews are available (Austin, 1961; *Bibliography of Reproduction,* 1970). Recent experiments on the transfer of embryos of farm animals, either nonsurgically via the cervical canal or surgically into the uterus include Sugie et al. (1965), Moore (1968), Rowson et al. (1969, 1971), Pope and Day (1970), and Testart and Leglise (1971).

Mouse eggs will develop from the two-celled stage at pH levels between 5.8 and 7.8 (Whitten, 1956; Brinster, 1965a). The optimum pH depends on the concentration of pyruvate or lactate in the culture medium, and a pH level of 7.2-7.4 is usually used. An optimum osmolarity of 276 mosmols was recommended by Brinster (1965a) for embryos between the 2-celled stage and the blastocyst, but 256 mosmols permitted development from the one-celled stage to blastocysts (Whitten and Biggers, 1968). More recently, optimal development of mouse ova from the pronuclear stage to blastocysts occurred with an osmolarity of between 250 and 280 mosmols and a pH of 7.2-7.3 (Whitten, 1971). Most embryologists use a gas phase of 5% CO_2 in air or 5% CO_2, 5% O_2, and 90% N_2 (Brinster, 1970c; Whitten, 1971).

TABLE 6–*Some References on the Fertilization and Cleavage of Mammalian Ova in Vitro, Followed by Embryo Transfer into Recipient Females*

Species	Reference
Rabbit	Chang, 1959
	Bedford and Chang, 1962
	Thibault, 1969
	Brackett, 1969b
	Seitz et al., 1970
Mouse	Whittingham, 1968
	Mukherjee and Cohen, 1970
	Cross and Brinster, 1970

Initial studies showed that mouse embryos of less than eight cells needed serum albumin or a complement of amino acids in the medium as a nitrogen source, although many of the essential amino acids, with the possible exception of cystine, could be omitted without affecting development into the blastocyst (Brinster, 1965c). Mouse embryos will actually develop from the two-celled stage into blastocysts, and then to full term on transfer to a recipient female, in the absence of a fixed nitrogen source (Cholewa and Whitten, 1970). Endogenous stores in the early embryo were evidently sufficient for cleavage and normal development.

The early mouse embryo will not survive if glucose is the only energy source; pyruvate and perhaps lactate must be supplied in the culture medium (Brinster, 1971b). More glucose carbon than pyruvate carbon is incorporated into the preimplantation stages of the mouse embryo, although more pyruvate is oxidized (Brinster, 1967b, 1969). Thus the culture requirements of the cleavage stages of the mouse are simple but specific; the embryos grow best in a medium with pyruvate as an energy source and a nitrogen source such as bovine serum albumin or amino nitrogen.

Information on the requirements of embryos of other species is not so extensive. The cleaving rabbit embryo is not dependent on exogenous energy sources, but both pyruvate and lactate are beneficial and are oxidized to CO_2 throughout the preimplantation period (Brinster, 1970b, 1970c). Monkey oocytes preferentially oxidize pyruvate rather than glucose and appear to be metabolically similar to rabbit embryos in this respect (Brinster, 1971b). Rabbit embryos evidently require an amino nitrogen source; there is some debate about the necessity of including a variety of amino acids (Daniel and Olson, 1968; Maurer et al., 1968; Brinster, 1970b).

Species variations exist in the active metabolic pathways in oocytes and embryos. In the rabbit embryo, more CO_2 is formed from carbon 1 of glucose than from carbon 6, indicating that glucose is used preferentially via the pentose shunt (Fridhandler, 1961; Brinster, 1967a, 1968c). In the mouse embryo, a ratio of C_1 to C_6 utilization close to one suggests that oxidation is predominantly by the Krebs cycle (Fridhandler, 1961; Brinster, 1967a, 1968c), but there is now some question about the value of C_1:C_6 ratios for the identification of metabolic pathways (Mahler and Cordes, 1966). Little is known about primate oocytes, although the human oocyte contains relatively low levels of LDH in comparison with some other oocytes (Brinster, 1968a).

Changes in enzyme activity or metabolic pathways could reflect alterations in the permeability of the embryo to various substrates. No significant difference in glucose permeability could be found between two- and eight-celled mouse embryos (Wales and Brinster, 1968). On the other hand, permeability of the embryo to malate, citrate, and α-oxyglutarate shows a considerable increase between the two- and the eight-celled stage, which may explain the increasing

activity of the Krebs cycle at the eight-cell stage (Wales and Biggers, 1968; Biggers, 1971). Unfortunately, changes in permeability and enzyme activity are difficult to evaluate since intermediates may be formed in the medium during the relatively long incubation period (Wales and Whittingham, 1970). The intracellular forms of the radioactive substrates originally in the medium are also difficult to determine because so little tissue is available. Alterations in the metabolism of cleaving embryos may arise through changes in mitochondrial structure that influence metabolic relationships between mitochondria and the cytoplasm (Biggers, 1971).

Cleavage of Human Embryos in Vitro

Initial studies on human embryos were carried out using the defined media developed for mouse embryos (Edwards et al., 1970). Certain parameters were standardized, e.g., a pH of 7.3, whereas others were varied, e.g., the gas phase and the osmotic pressure of the medium. In later studies, more complex media such as Ham's F 10 supplemented with human or fetal calf serum were utilized (Table 7) (Edwards et al., 1970; Steptoe et al., 1971). Ova rarely cleaved beyond the eight-celled stage in media designed for mouse embryos, despite alterations in the gas phase or osmotic pressure. Low osmotic pressure resulted in abnormal cleavage and fragmentation of the embryos. The most successful medium tested was Ham's F 10 supplemented with fetal calf serum, using a pH of 7.3, and osmotic pressure of 295-300 mosmols/kg, and a gas phase of 5% O_2, 5% CO_2, and 90% N_2. In this medium, cleavage was regular, the blastomeres being even-sized (Fig. 4).

The timing of cleavage is shown in Table 8, and examples of cleaving human ova are illustrated in Fig. 4. Several embryos differentiated into blastocysts after 147 hours or less in culture. The inner cell mass was prominent, the blastocoelic

TABLE 7–*Media Used for the Cleavage of Human Embryos in Vitro**

Medium†	No. of embryos	Osmolality (mosmol/kg)	Final no. of cells in embryo		
			1-4	5-8	>8
Whittingham's (1971a)	11	280-290	2	8	1‡
Whitten's (1971)	5	270	2	1	2‡
Waymouth's MB 752/1, with fetal calf serum	5	325	2	3	–
Ham's F 10, with fetal calf serum	17	295-305	0	1	16

* From Edwards et al. (1970).

† The pH of the media was 7.3-7.4.

‡ Cleavage after the eight-cell stage was irregular.

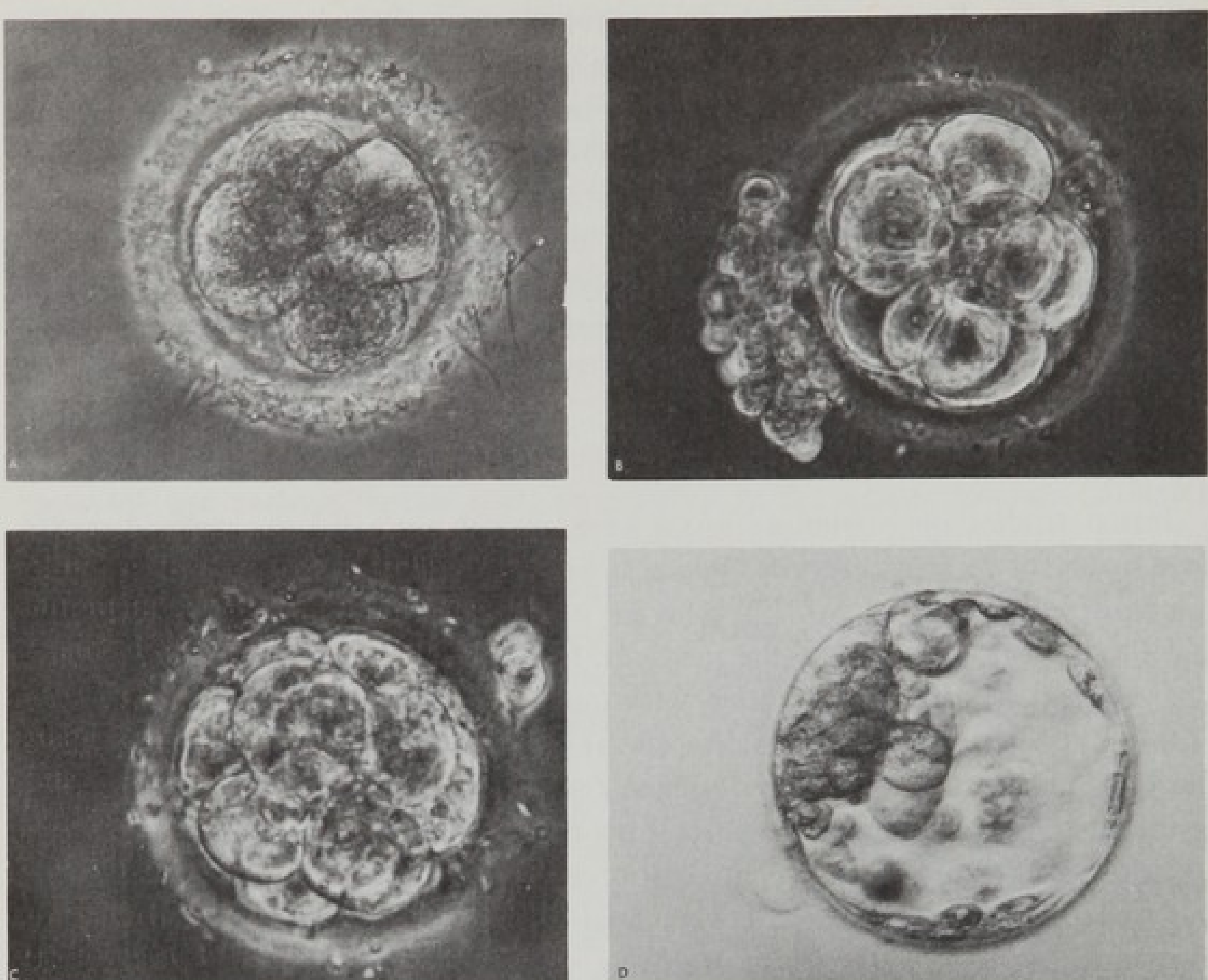


FIG. 4.–Cleavage in living human embryos. (A) Four celled. (B) Eight celled. (C) Sixteen celled. (D) Blastocyst: notice the secretory cells which first appear in the morula, the prominent inner cell mass, and the single layer of cells in the trophoblast. (Figs. 4A and B by courtesy of the editor of *Nature*.)

cavity occupied the majority of the volume of the embryo, and the trophoblast formed a single-celled layer around the embryos. Secretory cells appeared to be present in the trophoblast. More than 100 nuclei and many mitoses were found in the blastocysts after fixing and staining (Figs. 5A and 5B) (Steptoe et al., 1971).

TABLE 8–*Timings of the Cleavage of Human Embryos in Vitro*

Stage of development	Time (hours after insemination)
2-cell	<38
4-cell	38-46
8-cell	51-62
16-cell	<85
Morula	111-135
Blastocyst	123-147

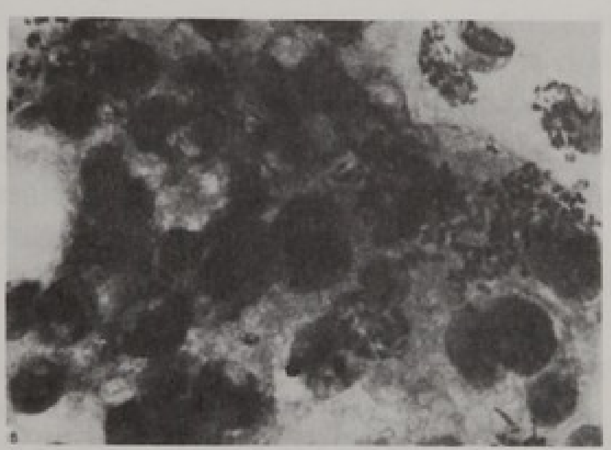


FIG. 5.–(A) A human blastocyst after staining; there are many nuclei and mitoses. (B) A detail from the inner cell mass region. (Fig. 5A by courtesy of the Editor of *Nature.*)

Cleavage of a human embryo to blastocyst was also reported by Shettles (1971), using a medium based on cervical fluid collected from human patients. Some doubt about cleavage must arise, however, for the embryo was not stained and there was no evidence that nuclei were present in this blastocyst. Cleavage of human embryos from the 2-cell to 12-cell stage occurred in Ham's F 10 supplemented with human serum and sodium estrone sulfate (Seitz et al., 1971). However, cleavage was irregular and often abortive; attempts to develop a repeatable in vitro system may have been unsuccessful since these investigations were using human oocytes which had been matured in vitro.

It is interesting to compare the growth of human embryos in vitro with development in vivo. A four-celled egg taken from the oviduct was estimated to be 48 hours postovulation (Doyle et al., 1966). A human blastocyst was flushed from the uterus approximately 6 days after the estimated time of ovulation (Croxatto et al., 1972). It was expanding and possessed approximately 150 nuclei. Two cell types were found in the trophoblast region, the typical trophoblast cells and a larger diffuse cell. Details of other human embryos recovered from the oviducts and uterus are summarized by Hamilton et al.

(1952) and by Mastroianni and Noriega (1970). The rates of development are thus similar in vitro and in vivo; it will be fascinating to find out if the secretory cells found in blastocysts grown in vitro correspond to the diffuse cells described by Croxatto and his colleagues.

Biochemistry of Preimplantation Development

The spermatozoon appears to provide a stimulus to continued development of the ovum, to donate a pronucleus, and perhaps a centriole, but little more. The cytoplasm of the spermatozoon, including its mitochondria and cytoplasm, is incorporated into the egg. The genotype of the spermatozoon influences the rate of cleavage of the embryo (Whitten and Dagg, 1961).

Large amounts of DNA are found in the ovulated oocyte, indicating that some of it is extrachromosomal (Reamer, 1963). The total amount of DNA increases during early cleavage, although the amount per cell decreases toward the nomal diploid quantity (Brinster, 1971a). DNA synthesis occurs during the pronuclear stages and during interphase of the cleavage divisions (Sirlin and Edwards, 1959; Mintz, 1964a; Piko, 1970).

Constitutive heterochromatin has been identified in eight-celled hamster embryos, as judged by its typical condensation and late replication (Hill and Yunis, 1967; Lee and Yunis, 1971). The quinacrine dyes or Geimsa reveal specific areas of chromosome banding in somatic cells (e.g., Gagńe et al., 1971; Sumner et al., 1971), although no fluorescence was found in most human embryos examined by fluorescence microscopy (unpublished data with P. L. Pearson and M. Bobrow). This lack of fluorescence, even in regions adjacent to the centromeres, resembles the weak staining of spermatogonia (Pearson and Bobrow, 1970).

Much RNA is also found in newly ovulated mouse ova despite the small number of ribosomes (Reamer, 1963). The total amount of RNA in a mouse embryo decreases rapidly up to the 16-cell stage so that each blastomere contains only a fraction of the RNA content of the ovulated egg. Yet studies on the uptake and incorporation of labeled uridine into mouse embryos show that some RNA is synthesized during this period, the rate increasing sharply during the eight-celled stage (Monesi and Salfi, 1967; Tasca and Hillman, 1970). The use of specific inhibitors has revealed that RNA synthesis is DNA-dependent during the cleavage of mouse embryos (Monesi et al., 1970; Tasca and Hillman, 1970). Various classes of RNA, including high and low molecular weight fractions are synthesized from the two-celled stage to the blastocyst (Ellen and Gwatkin, 1968; Woodland and Graham, 1969; Murdoch and Wales, 1971), although embryos at the four-celled stage, and later, synthesize mostly ribosomal and soluble RNA (Woodland and Graham, 1969; Piko, 1970). The RNA content of blastomeres in the morula approaches that of other tissue cells (Brinster, 1971a).

The incorporation of labeled amino acids also increases in eight-celled mouse embryos, although not as greatly as the precursors of RNA. Translation evidently occurs from both maternal and the newly synthesized RNA, and has been shown by measuring the rate of protein synthesis when RNA synthesis was blocked by actinomycin D. Protein synthesis continued on a reduced scale for some time, indicating that the RNA present before fertilization might be active in translation (Monesi et al., 1970; Tasca and Hillman, 1970).

The level of activity of certain enzymes alters during cleavage (Table 9). Lactate dehydrogenase (LDH) comprises as much as 5% of the protein in the mouse oocyte, which implies that this enzyme might be important in the growth and development of the oocyte (Brinster 1965b, 1971a). Levels of G-6-PD in mouse oocytes are also high when compared with other tissues (Brinster, 1966a; Epstein, 1970). The quantities of both LDH and G-6-PD decline rapidly during cleavage and then rise again in the blastocyst. There is much less LDH in the human oocyte than in the mouse oocyte (Brinster, 1968a; Epstein et al., 1969).

The activity of some enzymes, e.g., malate dehydrogenase and isocitrate dehydrogenase, increases slightly between the oocyte and the blastocyst in

TABLE 9–*Enzyme Activity During the Development of Preimplantation Embryos**

1. Activity increases
Hexokinase
Fructose-1,6-diphosphate aldolase
Malate dehydrogenase
Hypoxanthine-guanine phosphoribosyl transferase
Adenine phosphoribosyl transferase
2. Activity decreases
Lactate dehydrogenase
Glucose-6-phosphate dehydrogenase
Glutamic dehydrogenase
3. No change in activity
Isocitrate dehydrogenase
4. Activity increases and then decreases
Glycogen synthetase
5. Activity decreases and then increases
Aspartate amino transferase

* From Biggers (1971).

mouse embryos (Brinster, 1966b; Epstein et al., 1969). According to a more recent report, the activity of isocitrate dehydrogenase does not change during cleavage (Donahue and Stern, 1970). The activities of other enzymes increase markedly during cleavage in the mouse embryo: hexokinase (Brinster, 1968b), hypoxanthine-guanine phosphoribosyl transferase (HGPRT), and adenine phosphoribosyl transferase (Epstein, 1970). Changes in the activity of an enzyme are not necessarily related to its concentration, and measurements of the amounts of enzyme protein have not been done. The activation of preexisting enzyme, reduced degradation, or increased synthesis could all be involved in the increasing enzyme activity. The underlying causes of alterations in the activity of enzymes are obviously concerned with the relative influences of maternal and embryonic factors during preimplantation development.

Inactivation of One X Chromosome in Female Embryos (the Lyon Hypothesis)

Both X chromosomes in female germ cells are in an extended state, and human oocytes possess chromosomes similar to lampbrush chromosomes (Baker, 1971). Biochemical evidence also indicates that both X's are active in mouse oocytes, for XX oocytes contained twice as much G-6-PD activity as XO oocytes (Epstein, 1969). This conclusion must be tentative because G-6-PD has not yet been shown to be sex-linked in mice.

The typical condensation of one X chromosome occurs during cleabage or after implantation, depending on the species (Austin, 1966). A typical condensed, late-replicating X cannot be detected during cleavage of the hamster embryo (Hill and Yunis, 1967; Lee and Yunis, 1971). In rabbits, sex chromatin appears some 5 days after fertilization when a few hundred cells are present (Melander, 1962), although the late replication of one X can be detected 24 hours before the sex chromatin body (Issa et al., 1969). Formal proof that sex chromatin occurred only in female blastocysts was provided by Gardner and Edwards (1968) during experiments on the sexing of rabbit embryos. In the pig embryo, sex chromatin appears around the 50-cell stage (Axelson, 1968), whereas in mice and rats it does not appear until after implantation (de Mars, 1969). The late-replicating X chromosome is also found after implantation in rats (Lee and Yunis, 1971). Small pieces of condensed heterochromatin resembling sex chromatin were tentatively identified in human blastocysts (Steptoe et al., 1971).

The appearance of sex chromatin is presumed to be related to the inactivation of one X chromosome in cells of female embryos (Lyon, 1970). The mechanism and consequences of X-inactivation have been the subject of recent debate (Grüneberg, 1971; Lyons, 1971). An unsolved central issue concerning inactivation concerns the activity of the two X chromosomes in the early embryo before one of them is inactivated. Sex-linked marker enzymes would be

expected to provide the necessary information, but difficulties arise with this approach owing to the inheritance of "maternal" enzyme from the oocyte. Thus, G-6-PD can be detected in the early embryo, but most of it is evidently maternal in origin. A bimodality in levels of G-6-PD would be expected if the enzyme was determined by the X chromosomes of male and female embryos. No bimodality could be detected, perhaps owing to the swamping effect of maternal enzyme (Brinster, 1970a; Daentl and Epstein, 1970). In two-celled embryos, levels of HGPRT were similar in XO and XX embryos, perhaps due to the levels of this enzyme inherited from the oocyte, but by the morula stage the activity of the two types of embryo was related to their X-chromosome complement (Epstein, 1971; Epstein and Daentl, 1971). As Epstein points out, however, activity is not synonymous with levels of enzyme, and the gene HGPRT is not known with certainty to be sex-linked in mice. Nevertheless, Ohno (1967) has argued that genes on the X chromosome are conserved in mammalian species, and HGPRT is sex-linked in man. Conclusions about the activity of HGPRT reflecting transcription from X chromosomes in female mouse embryos must therefore remain tentative.

Embryological tests correlate well with the cytological observations on the time of appearance of the sex chromatin body. Gardner and Lyon (1971) have injected a single embryonic cell carrying X chromosome markers into a recipient blastocyst in order to determine when X-inactivation occurred in mice. Distinct coat color markers determined by the two X chromosomes were used to decide if X-inactivation had occurred at the time of injection. They concluded that X-inactivation had not occurred in the 3½-day blastocyst, and more recent work indicates that inactivation has still not occurred at 4½ days (Gardner, 1971; Lyon, 1971).

Both alleles of certain X-linked genes might remain active for some time during human fetal life and not display dosage compensation (Steel, 1970). Steel found that G-6-PD activity was significantly higher in erythrocytes and fibroblasts of female fetuses and newborns than in males. In contrast, HGPRT activity was identical in fetuses and newborns of both sexes.

Frozen Storage of Mammalian Embryos

Various attempts have been made to freeze-store mammalian embryos. Brief chilling was compatible with the further development of rabbit embryos (Chang, 1950) and mouse embryos (Whittingham and Wales, 1969). Fertilized rabbit and unfertilized mouse eggs have developed to full term after freezing in protective media (Ferdlows et al., 1958; Sherman and Lin, 1959). Recently, a high proportion of eight-celled mouse embryos and blastocysts survived and developed to full term after being frozen-stored at −79°C for 30 min and then thawed in medium containing polyvinylpyrrolidine (Whittingham, 1971b).

Blastocysts withstood freezing better than eight-celled embryos as judged by their ability to develop in vitro and in vivo. The success of the freezing technique is shown by the high proportion of embryos developing after freezing; 70% of the eight-celled eggs and 91% of the blastocysts appeared to be normal after recovery. Following transfer into foster mothers, 21% of the eight-celled eggs and 70% of the blastocysts developed into viable fetuses (Whittingham, 1971b).

CELLULAR REGULATION IN PREIMPLANTATION EMBRYOS

Regulation of Blastomeres

The blastomeres of cleaving embryos are highly labile and not predetermined to form particular embryonic structures. Destruction of all but one blastomere in two-celled, four-celled, or even eight-celled embryos can be compatible with the development of the remaining blastomere into a viable full-term fetus (Nicholas and Hall, 1942; Tarkowski, 1959; Tarkowski and Wroblewska, 1967; Moore et al., 1968; Mulnard, 1971). A considerable degree of lability remains in the blastocyst. Destruction or withdrawal of large parts of the inner cell mass is compatible with continued embryonic development to full term (Lin, 1969), and partial blastocysts obtained by sectioning can also reconstitute and develop to full term (Gardner, 1971). Cellular regulation is obviously possible even when RNA and protein synthesis is very active, and when overt differentiation is well underway.

Explanations of the embryological factors involved during cytodifferentiation in early development have been plentiful, but have lacked critical experimental support. Chemical gradients in the cytoplasm of the oocyte were thought to establish an initial polarity, successive cleavages parceling out these cytoplasmic areas into different blastomeres (Dalcq and Seaton-Jones, 1949). Results accruing from the experimental destruction of blastomeres were taken to support this concept, for the remaining blastomeres were believed to exhibit a polarized development into inner cell mass or trophoblastic tissue (Tarkowski, 1959). However, later experiments gave results which could not be explained in this way. Two or more eight-celled embryos could be fused together and develop into fetuses, with blastomeres from the two embryos reaggregating at random (Mintz, 1964a, 1965). A great deal of data have accumulated on the experimental fusion of cleaving embryos (e.g. Tarkowski, 1961, 1969, 1970; Mintz, 1968, 1969). Fusion followed by regulation can still occur at the 16-cell stage, and with less success when the embryo has between 16 and 32 cells. The considerable degree of lability in early development was well illustrated by the fusion of 16 morulae into a single embryo (Mintz, 1971b). Other observations by Mulnard (1965), Tarkowski and Wroblewska (1967), and Moore et al. (1968) also show that the embryo remains labile until almost the blastocyst stage. A

hypothesis known as "in/out differentiation" has been proposed to explain initial differentiation; according to this hypothesis, blastomeres separated from the external surface acquire embryonic determination and form the precursors of the inner cell mass, whereas those on the periphery differentiate into trophoblast (Tarkowski and Wroblewska, 1967; Mulnard, 1971; Graham, 1971). Attempts to confirm this hypothesis have been made by marking central and peripheral cells with inert silicone fluid (Wilson et al., 1972). If there are also secretory cells in the trophoblastic area of the human trophoblast (see p. 78), such a simple explanation of early development would hardly suffice to explain the origin of three cell types. The recent introduction of rapid tests for the alleles of β-glucuronidase and glucose phosphate isomerase (Condamine et al., 1971; Chapman et al., 1971) should facilitate the critical analysis of "inside/outside" differentiation by providing genetic markers to type cell lineages in chimeric embryos.

The inner cell mass might retain a role in the differentiation and proliferation of trophoblast (Gardner, 1971). The trophoblast present in the blastocyst is unlikely to be the precursor of the later fetal membranes, for it is probably already differentiated and merely retains a restricted capacity for development into giant cells.

Experimental Chimeras

Spontaneous mosaic or chimeric embryos are relatively rare (see p. 72) and restricted in their array of genetic markers which can be usefully employed in embryonic studies. Some of these limitations have been overcome by the artifical creation of chimeric embryos, and the method has now become a powerful tool in the analysis of mammalian embryology. The most widely used technique has been to fuse two cleaving embryos, for the resulting embryo will develop normally when transferred to a recipient female. The two stemlines resulting from the original embryos are found in most or all tissues of the body. Such animals, called allophenic mice by Mintz, have been used for a wide variety of studies encompassing many aspects of embryogenesis.

Chimeras are proving valuable in studies on sexual differentiation. In the original paper, chimeras resulting from the fusion of a male and a female embryo were found to differentiate mostly as males (Tarkowski, 1961), and these observations were confirmed by Mystkowska and Tarkowski (1968) and McLaren and Bowman (1969) (see Table 10). On the other hand, Mintz (1968) found the sex ratio of such chimeras to be 1:1. The discrepancies between these observations could be due to the degree of chimerism in the embryos (Mullen and Whitten, 1971): if one cell line dominated in the chimera, the sex ratio was unity, but where neither cell line predominated the sex ratio approached 3:1. Many cases of human intersexuality or hermaphroditism have been ascribed to whole-body chimerism, but Benirschke (1970) accepts only six of them as

TABLE 10–*Sex Ratios in Chimeric Mice**

Source	Male	Hermaphrodite	Female	Sex ratio
Tarkowski (1961)	11	3	2	4:1
Mystkowska and Tarkowski (1968)	17	1	6	
McLaren and Bowman (1969)	13	0	1	
Mintz (1968)	241	6	216	1:1
Mullen and Whitten (1971)				
One cell line dominant†	–	–	–	1:1
Neither cell line dominant†	–	–	–	3:1

* Modified from McLaren and Bowman (1969).

† Degree of chimerism judged from coat color.

proven; XX cells predominated in ovarian tissue and XY cells in testicular tissue, a strong XY preponderance favoring testicular development.

One early use of chimeric mice was the fusion of normal and t^{12}/t^{12} embryos; homozygous t^{12} embryos die in the morula stage. After fusion, t^{12}/t^{12} cells had a longer life-span and could be found in blastocysts (Mintz, 1964b, 1964c). Allophenic mice have yielded information on cytodifferentiation. After fusing two embryos each carrying a different allele of isocitrate dehydrogenase, Mintz and Baker (1967) found a hybrid enzyme in the skeletal muscle of allophenic mice. This enzyme is a dimer, and the hybrid enzyme is not formed from mixtures of the pure forms. Identification of the hybrid enzyme was convincing evidence that this tissue arose by myoblast fusion whereas other tissues do not. Allophenic mice have also been used to study the differentiation of the mammalian pigmentary system (Mintz, 1967), the behaviour of germ cells in mice with sex chromosome mosaicism (Mintz, 1969; Tarkowski, 1969, 1970; Mystkowska and Tarkowski, 1968, 1970), and the histocompatibility antigens on melanoblasts and hair follicle cells (Mintz and Silvers, 1970). Male proteins can be secreted by XX cells in XX/XY allophenic mice (Mintz et al., 1972). Embryos differing from each other at nine genetic loci have been fused (McLaren and Bowman, 1969). Allophenic mice have also been invaluable in studying "clonal development" in mammalian embryos (see p. 89).

The fusion of embryos does not provide an effective control over the degree of mosaicism in different tissues, nor is it possible to regulate the number or type of donor cells contributing to the chimera. Alternative methods for obtaining mouse chimeras have been described by Gardner (1968, 1971). Donor cells from the inner cell mass of one blastocyst were injected into the blastocoelic cavity of another (Fig. 6). Extensive mosaicism of the skin and coat occurred when only three cells were transferred, and further refinement enabled a single donor cell to be transferred (Fig. 6) (Gardner, 1971; Gardner and Lyon, 1971). In some chimeras, much of the pigmentary system was derived from the

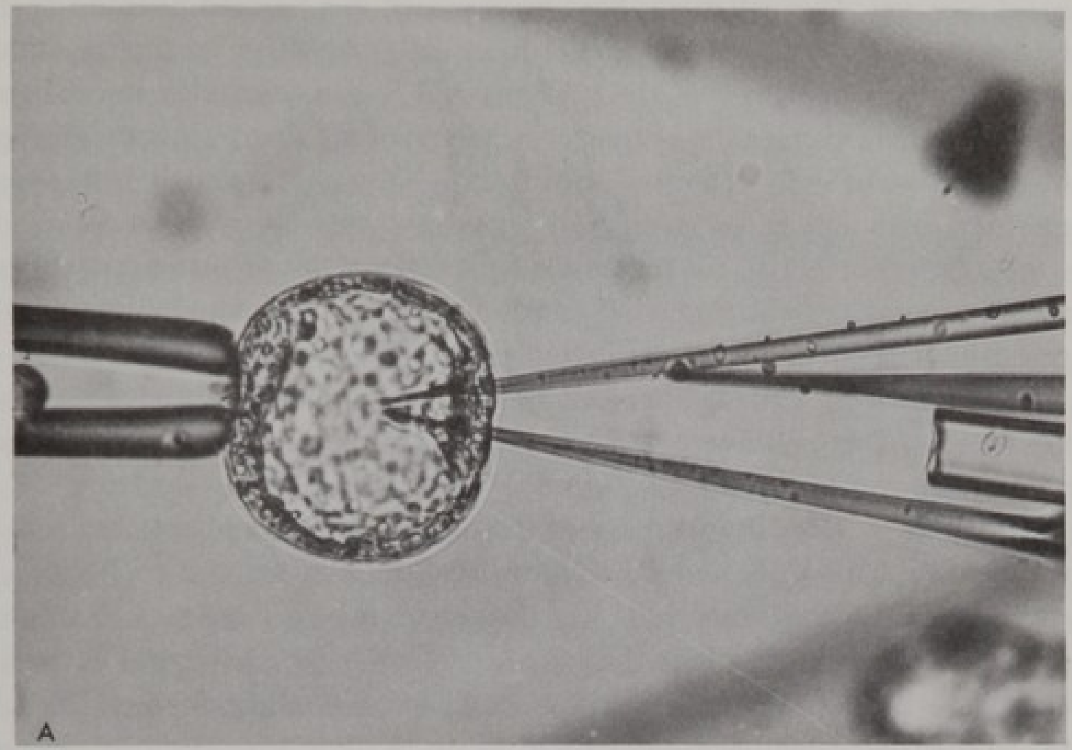


FIG. 6.–Injection of donor cell into a blastocyst. (A) The living mouse blastocyst is held in a suction pipette, and an aperture is made in the trophoblast by three needles. The small pipette contains the donor cells. (B) A chimera produced by injecting one cell carrying the genes for pigmented coat into an albino blastocyst. (We thank Dr. R. L. Gardner for permission to reproduce these illustrations.)

single donor cell. This cell might have been placed in a favored position or it may have been genetically superior to the host cells. Another possibility, that the donor cell was a precursor of the pigment cells, can be excluded since there was also considerable chimerism in the germ cells. At first glance, it is surprising that one cell can yield such extensive colonization. Observations such as this support suggestions that only a few cells—perhaps as few as three—are true precursors of the fetus (Mintz, 1971b), the remaining cells differentiating into extraembryonic tissues. It seems certain that new developments in producing chimeras will be aimed at exerting control over the colonization of specific organs.

The presence of two distinct cell lines in an embryo has various important consequences. One cell line could overgrow the other, so that varying ratios of the two cell types would be found in different tissues. Evidence of such overgrowth was found by Mintz and Palm (1969) in allophenic mice formed from the fusion of C3H and C57 embryos. Selection favored C57 cells in the erythrocyte cell lines but not in other organs, e.g., liver. An alternative explanation for these results is that the ratio of the two cell types in the initial cell pools varied considerably. But this explanation would not cover the data of Mintz (1968) on selection in the diploid phase of spermatogenesis in adult mice, for the initial ratio of the two types of offspring from allophenic males changed considerably and consistently with successive litters. The ratio of the two cell types in the testis was obviously changing with age, and this phenomenon could occur in other tissues. The control of meiosis and germ cell selection has recently been reviewed by Donahue (1972).

Evidence for the selective growth of particular cell lines in women was found by Nyhan et al. (1970). Two sisters who were double heterozygotes for alleles of G-6-PD and for HGPRT deficiency displayed only one cell line in their blood cells. This line carried the normal HGPRT allele and the G-6-PD allele linked to it in the *cis* configuration. The line carrying HGPRT deficiency was missing. In other tissues, both the cell lines were expressed. Another possible example of cell selection in man is the alteration in the ratio of normal to trisomic cells in children with normal/G trisomy mosaicism (Taylor, 1968, 1970). Why do these changes occur in the ratio of two cell lines? The rates of mitosis in the two cell lines could differ, or there could be subtle differences in their metabolism in different tissues. Another cause could well be "metabolic cooperation" between adjacent cells (Subak-Sharpe, et al., 1969; Subak-Sharpe, 1969). When cells in tissue culture are in close contact, a few normal cells can induce the production of enzymes by deficient cells so that there is a net increase in the amount of enzyme synthesized. The molecular entity transferred between adjacent cells might be a nucleotide. Not all cell lines show metabolic cooperation, but it has been demonstrated for hamster cell lines lacking inosinic pyrophosphorylase or adenylic pyrophosphorylase activity (Subak-Sharpe et al., 1966, 1969; Subak-Sharpe, 1969) and in human Lesch-Nyhan cells (Friedman et al., 1968),

although not for other cell lines. Phenomena such as metabolic cooperation could obviously be involved in allophenic mice, and especially in "solid" tissues. Numerous phenotypic effects could be associated with this type of phenomenon, perhaps even some form of hybrid vigor. The further study of allophenic mice should prove very rewarding in this respect.

Another opportunity arising from the development of fused embryos is that mutant phenotypes may be modified. The persistence of t^{12}/t^{12} cells after fusion with a normal embryo (Mintz, 1964c) could well be another example. A mixture of normal and mutant cells is reported to modify defects in cartilage formation caused by the recessive gene "short ear" in mice (McLaren and Bowman, 1969). On the other hand, the susceptibility of C3H cells to mammary tumors was evidently unchanged when C3H embryos were fused with C57 embryos (a low-susceptibility strain) (Mintz and Slemmer, 1969; Condamine et al., 1971).

A related question concerns the immunological relationships between the two cell lines in allophenic mice. Mosaic populations of erythrocytes are detectable, both cell types persisting throughout life (Mintz and Palm, 1969). Originally, a mutual state of "intrinsic" immunological tolerance was supposed to exist between the two cell lines in allophenic mice (Mintz and Silvers, 1970), but the situation now seems to be more complex. Each cell line evidently responds immunologically to the presence of the other and perhaps mutually with the mother, resulting in a defensive immunological system known as "enhancement facilitation": protective "blocking" antibodies protect each cell type from immune rejection by the other (Hellström et al., 1969; Wegmann et al., 1971). This form of protection is also extended to host cells in human chimeras formed when marrow grafts are given to agammaglobulinemic children (Jose et al., 1971).

Embryonic Cell Pools ("Clonal Development" of Organs) in Mammals

Evidence is accumulating to show that a few stem cells differentiate successively in mammalian embryos to form the primordia of major organ systems. Some of these primordia must differentiate very early in development, perhaps shortly after implantation. This form of differentiation has become known as the clonal development of organs although this is a misnomer in that several cells appear to contribute to all organ systems. Mosaic and chimeric embryos provide an elegant system for analysis, for the presence of two stemlines of cells in these individuals permits calculations to be made of their ratios in different organs. In man, sex-linked genes have proved valuable for this purpose (Nance, 1964; Nesbitt and Gartler, 1971) because X-inactivation results in mosaicism. The method of calculating the stem cell number in embryonic pools is as follows. If two embryos, A and B, were fused and a single stem cell

was the precursor of a particular tissue, then only one cell line (either A or B) could be present in the tissue. A population of animals would thus display A or B, with a frequency distribution of 1:1. With two stem cells, the characteristics of one or the other cell line, or both, would be displayed in different animals, with an expected frequency distribution of 1A:2AB:1B. The statistics become more complex with increasing numbers of cells, but frequency distributions can be calculated for each stem cell number. This type of analysis demands certain assumptions, e.g., that there is random mixing of the two cell lines, no selective migration of one cell line into the stem cell population, and no selective overgrowth of one cell line after differentiation. These assumptions are not always necessarily true (see Nesbitt and Gartler, 1971).

Observations on a population of human females heterozygous for alleles of G-6-PD showed that six cells or less formed the precursors of the hemopoietic system in man (Gandini et al., 1968), the estimates being based on an analysis of the ratio of heterozygous women with G-6-PD-deficient cells only. But these estimates are valid only if the same assumptions apply as in chimeric mice, and if X-inactivation occurred randomly before the differentiation of embryonic cell pools. The assumption concerning the time of X-inactivation might be correct as judged by the experimental evidence of the time of X-inactivation in various species (see p. 82). However, X-inactivation may not always occur at random in different cells, as found in women heterozygous for HGPRT deficiency (Nyhan et al., 1970). The X chromosome carrying HGPRT had been selectively inactivated in the hemopoietic tissue, which would imply that inactivation followed differentiation, or that selection had operated against the deficient cells during growth.

Allophenic mice can be used for a wider variety of studies, and there is no problem about the timing of X-inactivation. The most extensive studies have been carried out by Mintz (1964a, 1968, 1969, 1970). Using variants of glucose phosphate isomerase, serum allotypes of the 7S gamma globulins, and mutants of hair follicles and other tissues, she apportions 150 stem cells for the hair follicles, 20 stem cells for the photoreceptors of the eye, and 34 cells for the melanoblasts (Mintz, 1967). Mintz and Palm (1969) have shown the primordial erythrocytic pool to be three or four cells as judged by the analysis of C3H/C57 allophenic mice. Similar kinds of data were obtained by Wegmann and Gilman using markers on the 7S gamma globulins, beta-chain globulins, and coat color; they concluded that not less than five cells and probably more contributed to the hemopoietic and melanocyte systems in the mouse. The largest possible number of primordial germ cells in mice was calculated as nine, although the actual number may be smaller (Mintz, 1968). A very few cells in the blastocyst, perhaps only three, are the precursors of the embryo (Mintz, 1971b). This small number of "true" embryonic cells would explain why the injection of one to

three cells into the mouse blastocyst results in such extensive colonization of the fetus (Gardner, 1971).

SEXING OF PREIMPLANTATION EMBRYOS

Recent evidence has shown that the primary sex ratio in the mouse, and in other species, is close to 1:1 (Table 11). Attempts to separate X- and Y-bearing spermatozoa by a variety of techniques have not succeeded in consistently and significantly altering the secondary sex ratio (Beatty, 1970). Three approaches towards the sexing of preimplantation embryos have been described: identification of sex chromatin or the Y body in the nuclei of embryos, the attempted use of sex-linked enzymes, and the use of immunological methods. The work on enzymes can be mentioned briefly: no bimodality in levels of G-6-PD was found in mouse blastocysts by Epstein (1969) or Brinster (1970a), although levels of HGPRT could reflect the number of X chromosomes in the mouse morulae (Epstein, 1971; Epstein and Daentl, 1971).

Sex chromatin can be identified in the blastocysts of some species, and shortly after implantation in others (Austin, 1966). It is easily seen in rabbit blastocysts (Melander, 1962). Fluorescence methods were used to identify sex chromatin in intact rabbit blastocysts presumed to be female, but so reduced the viability of the embryos that none implanted (Edwards and Gardner, 1967). Small pieces of trophoblast can be excised from rabbit blastocysts and used for

TABLE 11–*Sex Ratios Soon After Fertilization in Various Mammals*

Species	Stage of embryos	Males	Females	Reference
Mouse				
Normal fertilization	3½-4½-day blastocysts	104	98	Vickers (1969a)
Delayed fertilization	3½-4½ day blastocysts	52	58	Vickers (1969a)
Hamster	Blastocysts	63	35	Sundell (1962)
Rabbit	Blastcysts	Ratio of one to one		Melander (1962)
Rabbit	Blastocysts	84	77	Gardner and Edwards (1968)
Pig	10-day blastocysts*	38	39	McFeely (1967)

Data from the rabbit based on sex chromatin and from chromosomal examination of embryos in the other species.

* Approximately 10% more embryos were chromosomally imbalanced.

sexing by staining for sex chromatin; the blastocysts recover from the operation in culture after a few hours. Many of the sexed blastocysts implanted in recipient females and all 18 living fetuses were found to have been correctly sexed (Gardner and Edwards, 1968). This was the first occasion when complete control over the secondary sex ratio had been achieved in any mammal. In species with small blastocysts, microsurgery can also be used for the removal of a few trophoblast cells, as shown in the mouse by Gardner (1968, 1971). Gardner suggests that the embryos tolerate the loss of a few cells because trophoblasts might be repopulated from the inner cell mass.

The expression of sex-linked antigens on embryos would be of considerable value in sexing embryos, although observations have been conflicting. The H-2 antigens were identified on preimplantation mouse embryos using mixed antiglobulin tests (Olds, 1968), and their presence on embryos was also deduced from the results of ectopic grafting of blastocysts (Simmons and Russell, 1966; Kirby et al., 1966). The opposite conclusion was drawn by Edidin et al. (1971) and Graziano and Edidin (1971), for ectopic transfers of mouse blastocysts did not sensitize the recipients. Later work also showed that the H-2 antigens could not be detected using direct tests on mouse embryos (Palm et al., 1971; Gardner et al., 1971), and antisera to the major histocompatibility antigens had no effect on the cleavage of mouse embryos in vitro (Heyner et al., 1969). The conclusion to be drawn is that transplantation antigens are not expressed on trophoblast. Mice do not become immunized to repeated injections of blastocysts (Billington, 1971), although there is some evidence that specific antigens exist on the trophoblast of monkey fetuses (Behrman, 1971).

Nevertheless, a recent paper indicates that there is a significant excess of male offspring in mice if the mother is splenectomized and then immunized against the Y antigen (Lappé and Schalk, 1971). The authors suggest that the immunological response after splenectomy permits male blastocysts to implant preferentially. But other reports of the preferential implantation of embryos differing widely from the mother have appeared and have been challenged by Palm (1970), who showed that a postnatal runting syndrome afflicts offspring similar to the mother, so leading to their selective mortality. This possibility must be excluded from Lappé and Schalk's results before their interpretation can be accepted.

USE OF PREIMPLANTATION EMBRYOS IN CLINICAL GENETICS

We will conclude this article by briefly mentioning a few of the clinical and scientific opportunities that could arise through the work described in this review. Many of the clinical advances associated with the maturation, fertilization, and cleavage of human embryos in culture are concerned with the alleviation of human infertility, reproductive physiology, and contraception. In

genetics, the applications of these methods should help us to understand the origin of some inherited disorders and assist in averting the birth of children with others. The control of early human development could also lead to the development of methods for modifying human characteristics. The ethical and social implications of this kind of work have been discussed elsewhere (Edwards and Sharpe, 1971; Edwards, 1971).

There is very little information currently available on the causes of various forms of anomalous human development. Many spontaneous abortions are chromosomally imbalanced, and detailed studies on meiotic and mitotic chromosomes in oocytes and cleaving embryos would supply data on the origins of various forms of aneuploidy. Information is needed on chiasma frequencies in oocytes with increasing maternal age, and on the related problem of recombination frequencies. This knowledge would enable us to decide if a "production line" of oocytes is gradually used up in the ovary as the mother ages. We also require information on the terminalization of chiasmata during the long dictyotene stage and during diakinesis, in order to decide if univalent formation does occur and thus lead to trisomy. Most studies on meiosis in oocytes have been carried out on mice, and these observations might not be relevant to man.

Studies are also needed on the effects of external agents on early development. Delayed fertilization is known to lead to triploidy in animals; yet there is no evidence on this point in man. Data could be obtained by deliberately delaying the fertilization of human oocytes in vitro. The effect of viruses on early development also requires analysis, especially in view of the repeated references to a viral etiology of mongolism. Nothing is known of the effects of drugs, X-rays, or other agents on human preimplantation development, and we must also gain knowledge on the radiosensitivity of these and other stages of development. The effect of contraceptive steroids on the chromosome complement of embryos requires more analysis. Nor do we know if human embryos can undergo delayed implantation, or respond to estrogens by an increased synthesis of nucleic acids and proteins as reported for animal embryos (Prasad et al., 1968).

In effect, a great deal of fundamental knowledge of these stages is required for practical and theoretical reasons. Although human embryos can be grown to blastocysts, the medium has not been highly defined and the biochemical pathways in the embryos have not been analyzed. There are unique opportunities for the study of embryological genetics: for example, two sex-linked mutants could prove of value in the study of X-inactivation in the human embryo. These are the heat-labile mutant of G-6-PD and the mutant of HGPRT causing primary hyperuricemia; cytochemical methods are available for detecting these enzymes in single cells (de Mars, 1968; Migeon et al., 1968; Rosenbloom et al., 1967; Salzmann et al., 1968). Sex-linked markers could provide information of the time of X-inactivation in the human embryo, by examining

heterozygous embryos for two populations of cells. In turn, this information will contribute to our understanding of embryonic cell pools in the embryo, for most of our information in man must come at present from the analysis of populations of women heterozygous for sex-linked genes.

Preimplantation embryos might also be useful for averting the birth of babies with genetic defects. One approach is to "type" preimplantation embryos for various characteristics and reject those found to be defective. This method would obviously be complementary to the current use of amniocentesis for the detection of human genetic disorders. A small piece of tissue would have to be removed from the embryo, and probably grown in culture to increase the number of cells available for analysis (Cole et al., 1966). The degree of cellular regulation existing during cleavage might prove of great advantage here, for animal embryos can tolerate a considerable degree of manipulation. Human embryos may be frozen-stored until the cell colonies have grown, for mouse embryos have already been stored for brief periods in this way. The difficulties should not be underrated, but they are not insuperable. These methods would be valuable where a segregating genetic defect was present in a family pedigree. Sex-linked disorders could be handled easily if sex chromatin or the Y body was detectable in embryonic cells, by simply replacing an embryo of the desired sex into the mother. Sufficient embryos must be available for analysis, and two or three embryos can already be obtained after a single laparoscopy.

Another potential method of alleviating genetic disorders is to place a few normal cells into a defective embryo. Microsurgical methods are successful with mouse embryos, and the fetuses are normal but chimeric. The first problem here lies in identifying the embryo at risk, and the second concerns the whole-body chimerism that would be an inevitable consequence with present methods. These methods would be radical and novel and need proving in species such as the mouse where there are sufficient mutant genes for study. Other advantages might be incidental to the treatment, for example some hybrid vigor arising through phenomena such as metabolic cooperation. The method could suffer from serious snags; e.g., the defects of both cell lines might be expressed in the offspring, one cell line might overgrow the other in time, and immunological complications might arise from the presence of two cell lines. Donor cells are being used therapeutically in various ways, for example in the cure of immunological deficiency disease in newborn animals and children (Pantelouris, 1971; August et al., 1970; de Koning et al., 1969; Gatti et al., 1968). Another "cure" of genetic defects might be to fuse the deficient cells with normal cells (gene complementation) and replace them in the donor, a technique mentioned by Schwartz et al. (1971) after they had corrected a deficiency of inosinic acid in mouse cells by fusing them with chick erythrocytes. Gene complementation has been used to correct complement deficiency in newborn mice (Levy and Ladda, 1971). Injecting donor cells or modified cells into blastocysts is probably the most radical form of this treatment.

Perhaps the most extreme form of embryonic manipulation is nuclear cloning, i.e., the replacement of the oocyte chromosomes with a nucleus from another cell. This method has been advocated as a means of conserving valuable human phenotypes. The method is untried in any mammal, and there are various embryological reasons why the claims made for it might not be achieved. Transferring nuclei in mammalian eggs could be a different proposition from amphibian eggs, for the mammalian embryo divides by a succession of full cell divisions whereas amphibian embryos undergo rapid cell divisions without recognizable G1 and G2 phases. Practically nothing is known about somatic mutations in man or animals, and a high rate of somatic mutation would negate the use of this technique for the conservation of valuable phenotypes. Nor is a great deal known about the effects of environment on human characteristics. The intrauterine environment can actually influence the number of vertebrae in a mouse fetus (McLaren and Michie, 1958). Obviously the prenatal, and probably the postnatal, environment could exert major effects on embryonic development. All these imponderables leave room for doubt that the "cloned" child would necessarily have the same attributes as the nuclear donor. We conclude that this method—which has been greatly overwritten as an immediate possibility—requires a great deal of proving before it can be considered a practicable means of conserving superior genetic individuals.

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CHAPTER 3

Heredity and Cancer in Man

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INTRODUCTION

The concept that cancer is a genetic disorder is not new. Lynch (1967) has called attention to a seventeenth-century proposal for a role for heredity in human cancer and to the many reports of familial cancer in the nineteenth century, but it has been primarily the investigators of the twentieth century who have developed fruitful ideas about genetics and cancer.

The principal contributions to these ideas have come not from a study of familial human cancer but rather from cytogenetic investigations and from studies of cancer in experimental animals. The former date back primarily to the work of Boveri and to the notion that chromosomal aberration is a primary event in carcinogenesis. However, it was chiefly the investigation of transplantable tumors in mice that led Tyzzer in 1916 to formulate clearly the concept of somatic mutation as a cause of cancer:

> There are marked differences in the behavior of various tumors on transplantation in given classes of mice. Even tumors arising in homogeneous races show such differences, and this may be attributed to the acquisition of new characteristics by the soma which are manifested in the development of the tumor. The tumor, since it breeds true with respect to these characteristics in the course of artificial propagation, may be regarded as a modification of the somatic tissue which may be termed *somatic mutation* [italics in original].

Since then, the somatic mutation hypothesis has had a stormy course but is still a favorite among oncologists, along with one which proposes a viral causation. Distinction between these hypotheses has become less meaningful as it has been demonstrated that the genomes of tumor viruses interact with the genomes of host cells. More attention has therefore been directed to means for testing consequences of a somatic mutation hypothesis.

A major consequence of the somatic mutation hypothesis is that individual tumors should be derived from single cells. That a single cell was able to produce malignant disease whereas crushed, nonviable cells did not was shown for mouse leukemia by Furth and Kahn in 1937. That human tumors often, even if not always, arise from a single cell has been demonstrated by two ingenious techniques in relatively recent times.

As Atkin (1970) has pointed out, chromosomal abnormality characterizes nearly all human cancers, except for numerous cases of acute leukemia and childhood cancers. These chromosomal abnormalities are not at all constant from case to case, but they are very similar from cell to cell within one tumor. Furthermore, there is a meaningful relationship among the karyotypes of these cells. Many of the cells in a tumor differ from selected other cells by a single chromosomal difference. These karyotypes can be arranged in a sequence of stepwise single changes, interpreted as the evolution of a stemline from a single cell. (For a review, see Atkin, 1970.) Analysis is complicated by artifacts of

preparation, so it is not possible to say that *every* cell is involved in such a clonal sequence. In some instances it is suggested that more than one stemline is present. This may result from the mixing of two different lines or from divergence from a common lineage. Second stemlines sometimes arise by polyploidization. Nevertheless, there is impressive cytogenetic evidence that many tumors arise from a single cell.

Another approach to this problem has been through the use of polymorphic X-linked loci. Taking advantage of the inactivation of one female X chromosome, which renders the cells of females mosaic for heterozygous loci (Lyon, 1962), Linder and Gartler (1965) demonstrated that individual uterine leiomyomata express only one of the alleles which specify glucose-6-phosphate dehydrogenase (G-6-PD). This analytical technique has also been applied to the important question of the origin of the Philadelphia chromosome (Nowell and Hungerford, 1960) in chronic myelocytic leukemia. This chromosome is found only in hematopoietic cells but is present in precursors not only of granulocytes but also of erythrocytes and platelets, but not of lymphocytes. Cells derived from precursors bearing the Philadelphia chromosome have only one form of G-6-PD, even when skin fibroblasts from the same individual contain two forms (Fialkow et al., 1967). From this it may be concluded that the Philadelphia chromosome arises only once. Fialkow (1970) has reviewed the evidence that other malignant states follow this pattern. There are some examples in which both alleles were active in one tumor. It is not ruled out, however, that noncancerous cells in tumors have given rise to the second type. Certainly there are numerous examples in which tumors have a definitely unicellular origin (Fialkow et al., 1970; Smith et al., 1971).

If cancer results from somatic mutation, how can we explain the hereditary tumors? What is the relationship between germinal and somatic mutation in the origin of cancer? Our purpose in this paper is to consider the role of germinal mutations in the origin of human cancer and to suggest a relationship between hereditary and nonhereditary forms of cancer.

GENETIC CONDITIONS PREDISPOSING TO CANCER

The aneuploidy usually associated with human tumors has played an important role in the development of a somatic mutation theory of cancer, and continues to do so (Ohno, 1971). Supporting evidence is provided by the observation that many carcinogens, including irradiation, some chemicals (Shaw, 1970), and some viruses (Nichols, 1966), cause chromosomal aberrations. However, the question arises whether malignant transformation is primary and aneuploidy purely secondary, whether aneuploidy is primary to malignancy, or whether a common agent may cause both aneuploidy and malignancy.

Unfortunately, this issue is not yet settled. The evidence noted above which

points to a single-cell origin of cancer suggests a primary role for aneuploidy. Furthermore, examination of premalignant lesions demonstrates the early establishment of aneuploidy (Atkin, 1970; Nowell, 1971a). Yet the abnormalities are generally nonspecific, with no single abnormality present in all cases of a given tumor. For many cases of acute leukemia and childhood tumors there may be no aberration demonstrable at any stage of the disease, although subtle changes of course cannot be ruled out. Also, chromosomal aberrations do not necessarily lead to malignancy, as they are observed in cases of viral infections (Nichols, 1966), exposure to irradiation, and exposure to various drugs or chemicals. Evidently the body ordinarily eliminates the aneuploid cells (Ohno, 1971). The persistence of aneuploid cells may reflect a failure in the immune system or the establishment of mutant cells with a selective advantage. Aneuploidy itself may aid in the progression of a neoplastic process (Ohno, 1971; Nowell, 1971b). Still, a direct causal relationship between acquired chromosomal aberration and malignancy cannot be established. Certainly agents which cause gross chromosomal breakage may alter the genetic activity of a cell in much more subtle ways which relate to the malignant process (Nowell, 1971b).

The only specific chromosomal change regularly associated with a human neoplasm is the Philadelphia chromosome in chronic granulocytic leukemia (CGL) (Nowell and Hungerford, 1960, 1961). This abnormality, found only in the hematopoietic cells and present in most cases of CGL, consists of a deletion in a G chromosome now identified by the fluorescent banding technique as number 22 (Caspersson et al., 1970). This abnormality apparently arises postzygotically, as it is found only in the target hematopoietic tissue. There are several case reports of monozygous twins in which only one member is affected and only he shows the Ph^1 chromosome (Dougan et al., 1966; Jacobs et al., 1966; Goh et al., 1967; Bauke, 1969; Kosenow and Pfeiffer, 1969; Keith and Brown, 1971). However, there is at least one instance of familial occurrence of CGL with the Ph^1 chromosome in monozygous twins and their sib (Tokuhata et al., 1968). Most remarkable is the family reported by Weiner (1965) and Hirschhorn (1968) in which several members (one a verified bearer of the Ph^1 chromosome) had died of CGL and numerous younger members were found to have the Ph^1 chromosome in their hematopoietic cells without CGL. In this family it appeared that the susceptibility for postzygotic tissue-specific chromosomal breakage was transmitted in an autosomal dominant fashion for three generations. Whether or not the younger individuals with the Ph^1 chromosome will develop CGL remains to be seen. The further transformation of CGL into a blastic phase has been attributed to the increased susceptibility of the aneuploid cell to acute leukemia, as the blastic crisis apparently occurs only in Ph^1 cells (Hirschhorn, 1968). The transition from normal to blastic phase apparently requires at least two steps.

The notion that cells with the Ph[1] chromosome constitute a population of aneuploid cells with a high susceptibility to transformation to acute leukemia brings up the problem of the risk of cancer in the aneuploid cell generally. What happens to the individual in whom most or all tissues are aneuploid, as when the error in cell division occurs in a germinal cell or early embryogenesis? If aneuploidy predisposes to malignancy, we expect an increased incidence of malignancy in those individuals with aneuploidy. If aneuploidy and malignancy have a common cause but neither is secondary to the other, we would expect to see an increased incidence of malignancy in both those with aneuploidy and in their cytogenetically normal relatives who experienced the same etiologic agent.

There are aneuploid states in which there is an increased incidence of leukemia. Most notable is Down's syndrome, with a 10- to 20-fold increase in the incidence of acute leukemia (Miller, 1970), and possibly a slight increase in incidence of solid tumors (Young, 1971). The increased risk applies to the translocation and mosaic forms as well as the usual trisomic form. Also the peak mortality from leukemia in Down's syndrome is younger by 2-3 years than in the general childhood population. Although fewer cases have been reported, there also appears to be an increased incidence of leukemia in trisomy D syndrome and in Klinefelter's syndrome (Fraumeni, 1969).

It has been well documented (Hecht et al., 1964; Day, 1966) that chromosomal abnormalities occur in a nonrandom fashion with respect to familial aggregation and temporal and spatial clustering. Also notable is the lack of specificity within the familial aggregations, such that Down's syndrome patients may have sibs with Klinefelter's syndrome or another trisomy, events not attributable to any simple parental gonadal mosaicism. Of further interest is the increase in incidence of leukemia in both the cytogenetically normal and abnormal members of such families (Miller, 1963). This association of leukemia and chromosomal abnormalities suggests that in such families there exists a common mechanism responsible for both the aneuploidy and the susceptibility to leukemia. Possible mechanisms suggested by Hecht et al. (1964) to account for the nonrandom occurrence of chromosomal anomalies also are pertinent to the association with leukemia, and include the following mutational events:

(1) Structural chromosomal rearrangements which predispose to abnormal chromosomal segregation could account for the increased tendency to aneuploidy noted in relatives of some aneuploids. Certain rearrangements could produce cells susceptible to leukemia. This possibility may soon be tested using new chromosome banding techniques (Drets and Shaw, 1971) which may recognize subtle rearrangements.

(2) The frequent presence of autoimmune disorders in relatives of aneuploid patients could lead to abnormal cell division and aneuploidy, and could be related to increased susceptibility to leukemia through immunologic disorder or through production of structural chromosomal rearrangements, as in (1). The

high incidence of thyroid antibodies and autoimmune disease in families of aneuploids has been related to a single gene defect transmitted as an autosomal dominant (Fialkow, 1966).

(3) Recessive mutant genes (meiotic mutants?) which increase the rate of chromosomal abnormalities could produce rearrangements leading to leukemia, as in (1).

(4) A virus or transmissible agent integrated into the cellular genome could produce familial aggregations of autoimmune disorders, chromosomal aberrations, and leukemia. Families would be identified only when some member is affected by a chromosomal aberration; however, the same mechanism could in some cases lead to familial aggregation of leukemia with no members affected by gross chromosomal aberrations.

A unique family with an inherited chromosomal anomaly, the Christchurch chromosome, a group G chromosome with a short-arm deletion, has been described in which 3 sib carriers of the abnormal chromosome developed chronic lymphocytic leukemia (Gunz et al., 1962; Fitzgerald et al., 1966; Fitzgerald and Hamer, 1969). However, association of the Christchurch chromosome and chronic lymphocytic leukemia may be fortuitous in this family, as there are numerous family members who carry the abnormal chromosome and are in the appropriate age group for the disease to develop, yet have no sign of disease. No other cases of chronic lymphocytic leukemia, familial or sporadic, have been found to harbor the abnormal chromosome.

In the above discussion the primary emphasis is on aneuploidy and leukemia, both in the same individual and in the same families with a possibly common etiology for the coexistent abnormalities. However, in some cases a chromosomal abnormality may relate to cancer in a different way. Aside from a general risk of leukemia in aneuploidy, certain specific chromosomal aberrations may affect a specific target tissue and predispose to cancer. In such cases there is no known increase in incidence of the specific tumor in other cytogenetically normal sibs. Specific examples include the association of trisomy 18 with multiple lesions resembling Wilms' tumor (Bove et al., 1969; Shanklin and Soteo-Avila, 1969) or with Wilms' tumor itself (Geiser and Schindler, 1969; Milligan and Young, 1971). It has been suggested that a regulatory gene could be located on chromosome 18 (Knudson and Strong, 1972a). Also, in 11 cases of the D-deletion syndrome, retinoblastoma occurred in 6, bilaterally in 5 (Taylor, 1970; Gey, 1970), suggesting that the gene locus for retinoblastoma may be on the long arm of a D chromosome.

Further examples of unusual chromosomal effect on specific target tissue may be seen in the association of cancer of the breast with Klinefelter's syndrome (Jackson et al., 1965; Dodge et al., 1969; Coley et al., 1971). Jackson et al. (1965) noted that patients with Klinefelter's syndrome had an incidence of cancer of the breast similar to that found in women, which may be related to the

abnormal hormonal balance in such subjects. A similar situation may be found in phenotypic females with a 46,XY karyogram or with sex chromosome mosaicism and a Y chromosome in dysgenetic gonads, who have a high incidence, 30-40% (Barr et al., 1967; Teter and Boczkowski, 1967) of gonadoblastoma or dysgerminoma, often bilateral (Talerman, 1971). XY gonadal dysgenesis may be familial even for sex chromosomal mosaics (Hsu et al., 1970). It is apparently transmitted through normal XX females, and an X-linked recessive or a sex-limited autosomal gene has been postulated (Scully, 1970a; Talerman, 1971). Tumor occurs much more rarely in patients with Turner's syndrome and has been reported only twice in ovaries with normal XX karyogram (Talerman, 1971). The predisposition to malignancy in these dysgenetic gonads may be related to a single gene mutation (familial form) or to the presence of a Y chromosome in an abnormal environment.

Consideration of genetic conditions which predispose to cancer must include the recessively inherited chromosomal breakage syndromes (German, 1972). These disorders apparently produce cells which carry on abnormal mitoses both in vivo and in vitro and have a high incidence of leukemia. Whether the defect is in the mitotic apparatus or in DNA synthesis or repair is not known. The most studied syndrome is that of Fanconi's anemia. In this condition the homozygote demonstrates a constant rate of chromosomal breakage (Hirschhorn and Bloch-Shtacher, 1970) at all ages and during all clinical stages of disease (Schroeder and Kurth, 1971). Chromosomal breakage and mitotic disorders have been observed in cultured fibroblasts and abnormal metaphases are observed on direct bone marrow examination. The chromosomal breakage thus seems to be both an "in vivo" and an "in vitro" phenomenon, present to a much less extent in the heterozygote. The site of the defect is not known. The heterozygote may or may not show increased chromosomal breakage (Hirschhorn and Bloch-Shtacher, 1970; Dosik et al., 1970; Visfeldt and Mortensen, 1970). There is an increased risk of leukemia in both homozygotes and heterozygotes (Garriga and Crosby, 1959; Swift, 1971). In fact, the proportion of Fanconi's anemia heterozygotes among all persons dying with leukemia has been estimated to be 1 in 20 (Swift, 1971).

Bloom's syndrome is often compared to Fanconi's anemia, as both demonstrate an increased rate of chromosomal breakage in vitro and in direct bone marrow preparations, abnormal mitoses in cultured fibroblasts (German, 1971; Schroeder and Kurth, 1971), and an increased incidence of leukemia. However, Bloom's syndrome differs from Fanconi's anemia in the production of symmetric quadriradial chromosomal configurations, as opposed to chromosomal breaks. In this disorder heterozygotes have a slight increase in chromosomal breakage in vitro, but the incidence of leukemia in them is not known. A normal rate of repair of ultraviolet-light-induced DNA damage has been reported for at least the first step of the repair process (German, 1972). In some cases there are

low levels of IgA or IgM and the affected children suffer an increase in frequency and severity of infection (German, 1972). Whether or not there is a primary defect in the immune system is not established.

A syndrome which may belong to the chromosomal breakage syndromes is ataxia telangiectasia. Approximately 50% of the patients of this recessively inherited disorder show chromosomal breaks similar to those found in Fanconi's anemia and Bloom's syndrome (Hecht et al., 1966; Schroeder and Kurth, 1971). In a series reported by Pfeiffer (1970), the 2 older patients (15 and 16 years) had chromosomal aberrations such as polyploidy, dicentrics, and abnormal chromosomes in cultured lymphocytes, and 2 young patients (3 and 5 years) had normal karyograms. Normal DNA repair of damage induced by ultraviolet light has been reported (Cleaver, 1968). Patients with ataxia telangiectasia suffer from defects in the immune system including variable immunoglobulin deficiencies (most often IgA and IgE), lymphopenia, and thymic hypoplasia. Lymphocytes fail to undergo blastic transformation in response to phytohemagglutinin (Gropp and Flatz, 1967). Although more than 50% of these patients die of chronic pulmonary disease, many of the remainder die of lymphoreticular malignancy such as acute lymphocytic leukemia, lymphosarcoma, and Hodgkin's disease. The high incidence of cancer may be related to the chromosomal breakage as in Fanconi's syndrome, or to the defect in the immune system. There is a striking tendency for sibs to develop the same type of cancer (Gatti and Good, 1971), indicating some specificity within families. There are reports of an increased incidence of cancer of the lymphoreticular system in families of patients with ataxia telangiectasia (Reed et al., 1966; Lampert, 1969), but others have not noted any increase (Gatti and Good, 1971).

Closely related to ataxia telangiectasia are the inherited immune deficiency disorders (Table 1) in which the age-specific incidence of cancer in young persons has been estimated to be 10,000 times as great as that in age-matched controls (Gatti and Good, 1971). However, rather than an increase in all types of cancer or in the most common cancers, as would be expected from a generalized lack of immunologic surveillance in eliminating neoplastic cells, there is a very high incidence of cancers of the lymphoreticular system, i.e., leukemia, lymphoma, Hodgkin's disease, malignant reticulosis, lymphosarcoma. The inherited disorders of the immune system then predispose primarily to neoplasia within the same system.

The most straightforward example of mutation and cancer may be the autosomal recessively inherited xeroderma pigmentosum, which is characterized by sensitivity to sunlight and by multiple cutaneous cancers. The primary defect in this disorder is in the genetically determined enzyme system necessary for DNA repair of ultraviolet light-induced damage (Cleaver, 1968, 1969). Chromosome studies on the lymphocytes of patients and heterozygous carriers are normal (Reed et al., 1969); however, cultured skin fibroblasts show occasional

TABLE 1.–*Immune Deficiency Disorders*

	Disorder*	Immune defect	Mode of inheritance	Increased incidence cancer
1.	Infantile X-linked agammaglobulinemia (Bruton type)	Absent humoral immunity; absent plasma cells; normal cellular immunity	Sex-linked recessive	Yes
2.	Di George syndrome (thymic aplasia, III-IV pharyngeal pouch disease)	Normal humoral immunity; absent cellular immunity; failure of third and fourth pharyngeal pouches	Unknown	None reported, but survival too short to ascertain
3.	Severe combined immunodeficiency (Swiss type, lymphopenic agamma globulinemia)	Absence of both humoral and cell-mediated immunity	Autosomal recessive or sex linked	Yes
4.	Wiskott-Aldrich disease	Deficient humoral immunity (low IgM); depressed cellular immunity; lymph adenopathy; failure to respond to polysaccharide antigens	Sex-linked recessive	Yes
5.	Isolated deficiency of IgA	Isolated deficiency of IgA	Autosomal dominant†	Probable, gastric adenocarcinoma‡
6.	Ataxia telangiectasia	Variable deficient humoral immunity (most often IgA); thymic hypoplasia; lympho-penia	Autosomal recessive	Yes (also may be increased inci-dence cancer of lymphoreticular system in relatives) 2 cases (sibs) of gastric adeno-carcinoma
7.	Common variable immunodeficiency (late onset, acquired dysgammaglobulinemia)	Decreased humoral immunity	Heterogeneous group–may be familial	Yes
8.	Chediak-Higashi § disease	Decreased ability to resist infection; giant leukocytic granules; probable lysosomal	Autosomal recessive	Yes

* Nomenclature from Gatti and Good (1971).

† State of homozygote not known.

‡ Data from Gatti and Good (1971); Tomkin et al. (1971).

§ Data from Good and Finsted (1969).

pseudodiploid clones (German, 1971) and an increased yield of chromosomal aberrations following low doses of ultraviolet light (Parrington et al., 1971). Even here, where the nature of the basic defect which predisposes to mutation and cancer is known, the site(s) of DNA damage which lead to cancer are not known.

In concluding a discussion of genetic conditions which predispose to cancer, notice should be taken of attempts to identify susceptible cells and populations. The most obvious conditions are those with gross chromosomal aberrations, followed by those with an increased rate of spontaneous chromosomal breakage. Attempts have been made to determine the sensitivity of such cells to various carcinogens, with the hope of expanding the study to detect cytogenetically normal cells with an increased susceptibility to cancer. Thus, lymphocytes and fibroblasts from Fanconi's anemia showed an abnormally great sensitivity to X-rays, as measured by chromosomal breaks per cell per rad (Higuraski and Conen, 1971). Studies with the chemical carcinogen benzpyrene have revealed that cells from patients with trisomy 21 or Fanconi's anemia show an increased percentage of endoreduplication and tetraploidy (Hirschhorn and Bloch-Shtacher, 1970). However, most such studies have utilized in vitro viral transformation of human cells, guided by the hypothesis that persons at high risk of leukemia and other cancers may be identified by the rate of oncogenic viral transformation of their fibroblasts (Miller and Todaro, 1969). The hypothesis demands that the fibroblast somehow reflect the risk of the hematopoietic or other target cell to develop cancer, implying an abnormality present in all cells but expressed in vivo only in specific cells. Use of this technique (Table 2) demonstrates that fibroblasts from Down's syndrome, trisomy 18, and Fanconi's anemia homozygotes and heterozygotes display high rates of viral transformation. The data on xeroderma pigmentosum are conflicting; although xeroderma pigmentosum may be a genetically heterogeneous disorder (Parrington et al., 1971), the report of Veldhuisen and Pouwels (1970) finding an increased rate of SV40 transformation in one patient as compared to his heterozygous mother has not been confirmed in further studies on related and unrelated patients (Aaronson and Lytle, 1970; Parrington et al., 1971). The immune deficiency disorders do not show any increased rate of transformation in preliminary studies (Gatti and Good, 1971). That the rate of transformation can be genetically determined was demonstrated by Todaro (1968), who found an apparently normal individual, his 2 siblings, and father with a high rate of transformation. Extension of the study to a family with several cases of sarcoma demonstrated a high rate of transformation in 3 persons. Snyder et al. (1970) reported a family with acute myelogeneous leukemia over three generations, normal chromosome analysis, and an increased rate of transformation in several members, including the proband, a sib, and their mother. Transformation was normal in the father and 2 brothers. Leukemic

TABLE 2.–*Relative Susceptibility of Cultured Fibroblasts from Selected Subjects to Transformation by SV40 Virus*

Classification of subject	Increase in susceptibility	Reference
Down's syndrome	3-5×	Miller and Todaro (1969)
Trisomy 18	6×	Miller and Todaro (1969)
Fanconi's anemia:		
Homozygote	10-50×	Miller and Todaro (1969)
Heterozygote	5-40×	Miller and Todaro (1969)
47,XXY (46,XY Mosaic)	3-10× (XXY line 3× >XY)	Mukerjee et al. (1970)
Sarcoma family	Increased	Todaro (1968)
Leukemia family	5-50×	Snyder et al. (1970)
Aged vs. young	Increased	Todaro (1968)
Xeroderma pigmentosum	13×	Veldhuisen and Pouwels (1970)
(genetically heterogeneous?)	Normal	Aaronson and Lytle (1970)
	Normal	Parrington et al. (1971)
Wiskott-Aldrich syndrome	Normal	Gatti and Good (1971)
Agammaglobulinemia	Normal	Gatti and Good (1971)
Triploidy	Normal	Schmickel et al. (1971)

relatives were in the maternal branch of the family, suggesting a correlation between the genetically determined high rate of in vitro transformation and the high incidence of leukemia.

This technique for identifying individuals at risk has been applied primarily to leukemia; however, further application to genetically determined susceptibility to cancers is clearly indicated. We could anticipate that in cases in which cancer susceptibility is determined by a germinal mutation an increased susceptibility to transformation might occur in every cell. A further expectation would be that cases in which the inherited susceptibility to cancer is due to a deficiency or absence of normal function in one tissue, other tissues being normal, there would not be any increase in transformation rate. This is the case in the immune deficiency syndromes. For xeroderma pigmentosum a high rate of transformation would be expected only in cells in which the genome had been modified by accumulated DNA damage.

If a common mutational event accounts for the nonrandom occurrence of chromosomal abnormalities and leukemia, then the susceptibility to leukemia should be manifest by an increased susceptibility to viral transformation in the cytogenetically normal family member as well as in the trisomic individual. We are not aware of reports on first-degree relatives of patients with chromosomal abnormalities and leukemia, but anticipate that such work would be fruitful for identification of individuals at risk for leukemia. The technique obviously might

lead to the early identification of those predisposed to cancer and to the elucidation of the mechanism underlying predisposition.

HEREDITARY TUMORS

The surest evidence that mutation, in the broad sense of inherited change, can play a role in the origin of cancer comes from the dominantly inherited tumors (Anderson, 1970). Here we shall consider the evidence that hereditary tumors are found in all organ categories and assess the relationship they bear to nonhereditary forms.

Embryonal

Probably the purest and best-studied example of a hereditary tumor is retinoblastoma. It is now evident that this childhood tumor does not always result from inherited mutation. There is a notable difference in incidence of affected offspring among the children of unilateral and of bilateral cases (Schappert-Kimmijser et al., 1966). In the latter the incidence among offspring is consistent with dominant inheritance, but in the former only 10% or so are affected. This difference does not appear to be attributable to two different mutations because the offspring of unilateral cases are often bilaterally affected and those of bilateral cases may be unilaterally affected. The most satisfactory explanation (Falls and Neel, 1951; Vogel, 1957) is that not all cases of retinoblastoma derive from germinal mutations but that some large fraction is somatic in origin.

The germinal mutation that causes all bilateral and some unilateral cases obviously does not cause tumor in every retinal cell, but rather creates a greatly increased probability that such a malignant transformation will occur. Some further change(s) must occur. A simple model has been created (Knudson, 1971) which hypothesizes that a second mutational event must occur in a somatic cell before tumor occurs. The nonhereditary cases are also conceived as resulting from two mutations, but with both occurring in somatic cells. All retinoblastomas are conceived as resulting from a two-mutation process, the difference between hereditary and nonhereditary forms being solely a matter of germinal or somatic occurrence of the first mutation. Germinal cases often have more than one tumor because every retinal cell harbors the first mutation. Somatic cases have acquired only one tumor because the probability that two rare events would both occur in two different cells in the same individual is immeasurably low.

Occasional individuals who inherit the first mutation do not develop any tumor; this fraction has been estimated to be in the range of 1-10% (Falls and Neel, 1951; Knudson, 1971). Gene carriers then may develop no tumor, one tumor, or more than one tumor. The distribution of numbers of tumors in gene carriers follows closely a Poisson expectation, with a mean number of 3. The

overall conclusion is that approximately 60% of all cases of retinoblastoma are nonhereditary and unilateral, 15% are hereditary and unilateral, and 25% are hereditary and bilateral (Knudson, 1971).

Not only are hereditary cases of retinoblastoma often bilateral but they also occur about 1 year earlier on the average than do nonhereditary cases. The ages at which the two forms occur have been shown (Knudson, 1971) to be distributed in keeping with one-somatic-hit and two-somatic-hit phenomena, respectively; i.e., only one somatic event is necessary to produce tumor in hereditary cases, as predicted by the two-mutation hypothesis.

Most children with retinoblastoma have no affected relative. Many of these are examples of the nonhereditary form, as defined above; the remainder may be presumed to result from new germinal mutations. Some children have a family history of the disease. In some of these one parent is affected, and the family cluster of cases is compatible with dominant inheritance. In other instances, however, both parents are unaffected. In a few rare instances the parent, as judged by occurrence of the disease in one of his parents, is an unaffected gene carrier, an example of nonpenetrance, as noted above. In typical instances, however, the only affected individuals in a pedigree are siblings. Several explanations of this phenomenon should be considered.

Perhaps the simplest hypothesis is that one parent is an unaffected gene carrier produced by new mutation. This possibility is rendered unlikely by the fact that the phenomenon is too common to be consistent with the observed penetrance in subsequent generations. A second possibility is that new mutation has occurred in an early stage in the germinal line in one of the parents, resulting in gonadal mosaicism and a significant probability that two or more offspring will acquire the mutation. This explanation would suffice for most of the atypical pedigrees, but not for all of them.

In some instances, the most remarkable collection being that published by Macklin (1960a), affected sibs are found in more than one branch of a pedigree, connected by too many unaffected individuals to be accounted for by the observed degree of nonpenetrance. This situation has been noted by Neel (1962) to parallel that of the lobster claw deformity, where Auerbach (1956) has offered the explanation, borrowed from studies on chemical mutagenesis in *Drosophila,* that unaffected carriers are carrying not the mutation itself but a premutation which, after a delay of one or more generations, becomes an expressed mutation, subsequently heritable in typical dominant fashion (Knudson, 1971).

One might argue that those tumors most likely to reveal characteristic Mendelian inheritance are the childhood tumors, and that retinoblastoma is but one of a class. Among the most common childhood tumors, however, this is not the case; only for some rare and special cases such as neurofibromatosis is it true. On the other hand, retinoblastoma is atypical among childhood tumors in that

the cure rate has been high enough for a long enough time that surviving and fertile affected individuals have produced affected offspring. If fertile survivors of other childhood cancers begin to appear, might they show a pattern of transmission like that of retinoblastoma?

If retinoblastoma patients had as poor survival as do other children with cancer, the principal manifestation of a hereditary form would be the occasional occurrence of tumor in sibs born to normal parents. Such a pattern of affected sibs has been observed for several childhood tumors, notably Wilms's tumor and neuroblastoma.

Some 58 reported familial cases of Wilms' tumor of the kidney have been reviewed (Knudson and Strong, 1972a) with this possibility in mind. In most of these 58 cases the affected individuals were siblings; in a few of these cases the sibs were identical twins. There were 3 instances in which tumor occurred in more than one generation and was compatible with dominant inheritance and incomplete penetrance. For this reason the mutation model developed for retinoblastoma was applied to Wilms' tumor as well.

Support for the model came from the observations that familial cases were much more frequently bilateral and were diagnosed at an earlier age than nonfamilial cases, just as with retinoblastoma, and in keeping with the mutation model. Furthermore, bilateral cases showed the same early age distribution that familial cases did. From the data it may be inferred that 35-40% of all cases of Wilms' tumor may be of the hereditary type. The mean number of tumors per gene carrier was estimated to be one, with the result that penetrance should be approximately 0.6, and carriers who are affected should have bilateral tumors in about 25% of cases.

A highly significant association has been observed which further supports a genetic origin for Wilms' tumor. This association is with aniridia (Miller et al., 1964). Aniridia itself is a dominantly inherited condition in which about 33% of all cases result from new germinal mutations (Shaw et al., 1960). Cases of aniridia show extreme variability with respect to associated congenital defects, some so severe as to be incompatible with survival to the age of reproduction. It is the sporadic (newly mutant) severe case which is at risk for Wilms' tumor; about 33% of such cases develop tumor (Fraumeni and Glass, 1968). It is possible of course that the basic growth defect in the disease somehow renders such children susceptible to Wilms' tumor, although no mechanism for such a phenomenon has been suggested. Another possibility is that severe cases of aniridia result from chromosomal deletions which include not only the aniridia locus but also adjacent genetic loci which relate to the associated defects and which include in some cases at least a locus controlling the development of Wilms' tumor.

A similar set of observations has been made for neuroblastoma (Knudson and Strong, 1972b). Review discloses 29 reported familial cases of this tumor,

showing a pattern like that found in Wilms' tumor. Most of the cases are sibs, some of these identical twins. In three pedigrees there was probable vertical transmission of either neuroblastoma or its pathologic relative, ganglioneuroma, compatible with dominant inheritance. Again familial cases occur earlier and are more often multiple than are nonfamilial cases. Although there were not adequate data to delineate an age distribution for nonfamilial bilateral cases, it is noteworthy that 15% of 40 congenital cases in one summary were bilateral (Schneider et al., 1965). From available data it was estimated that 20-25% of neuroblastoma cases occur in gene carriers (Knudson and Strong, 1972b). It was further estimated that the mean number of tumors which develops in gene carriers is approximately unity, giving the same penetrance and fraction of bilateral tumors as in Wilms' tumor.

For all three of these childhood cancers it is concluded that some fraction of cases occurs in individuals who are predisposed by a germinal mutation they have acquired. As shown for retinoblastoma (Knudson, 1971) the rate at which this germinal mutation (μ_g) occurs is a function of the incidence of the tumor (i), the fraction which is hereditary (f_h), the coefficient of selection (s), and the mean number of tumors (m), as follows:

$$\mu_g = \frac{f_h \cdot i \cdot s}{2(1 - e^{-m})}$$

Using reported values of incidence, the estimated values of f_h and penetrance $(1 - e^{-m})$, and the coefficient of selection shown, germinal mutation rates are shown for all three tumors (Table 3). All three rates are estimated to be in the range $5 - 19 \times 10^{-6}$.

The germinal mutation rate at the first locus affected in these tumors has been compared with the somatic mutation rate (μ_s) at the same locus in the nonhereditary (f_n) fraction in the case of retinoblastoma (Knudson, 1971), according to the following equation:

$$\frac{\mu_s}{\mu_g} = \frac{f_n}{f_h} \cdot \frac{1 - e^{-m}}{s} \cdot \frac{1}{m}$$

TABLE 3.–*Estimation of Germinal Mutation Rates for the Hereditary Forms of Three Childhood Tumors*

Tumor	Incidence (i)	Hereditary fraction (f_h)	Coefficient of selection (s)	Penetrance $(1 - e^{-m})$	Germinal mutation rate (μ_g)
Retinoblastoma	5×10^{-5}	0.40	0.5	0.95	5×10^{-6}
Wilms' tumor	10×10^{-5}	0.38	0.63	0.63	19×10^{-6}
Neuroblastoma	7×10^{-5}	0.22	0.63	0.63	8×10^{-6}

For retinoblastoma this ratio is essentially unity, i.e., the somatic mutation rate is equal to the germinal mutation rate. For Wilms' tumor and neuroblastoma the ratios are 1.6 and 3.5, respectively, although these values are much less precise than is the value for retinoblastoma.

Another variable of interest in these cancers is that of twin concordance. Concordance in identical twins should be equal to the product, fraction of hereditary cases (f_h) × penetrance ($1 - e^{-m}$) (Knudson and Strong, 1972a). The values for retinoblastoma, Wilms' tumor, and neuroblastoma should be 0.38, 0.24, and 0.14, respectively. Unfortunately, available data are scant. Published reports suggest that these values are too low, but the reports may be skewed in favor of concordance.

For no other common childhood tumors are there enough data to permit analysis of the type presented for retinoblastoma, Wilms' tumor, and neuroblastoma. The special cases of leukemia and lymphoma will be discussed below. Despite the rarity of other tumors, affected sibs, first cousins, and/or vertical transmission have been observed for the following tumors: brain tumors (type unspecified) (Miller, 1971), medulloblastoma (Leavitt, 1928; Griepentrog and Pauly, 1957; Kjellin et al., 1960; Bickerstaff et al., 1967; Belamaric and Chau, 1969), rhabdomyosarcoma in association with other soft-tissue sarcomas (Howard and Casten, 1963; Remzi and Kendi, 1966; Li and Fraumeni, 1969a, 1969b), osteogenic sarcoma (Roberts and Roberts, 1935; Pohle et al., 1936; Harmon and Morton, 1966; Robbins, 1967; Epstein et al., 1970; Bottomley et al., 1971; Miller, 1971), Ewing's sarcoma (Huntington et al., 1960; Hutter et al., 1964), adrenocortical carcinoma (Fraumeni and Miller, 1967; Kenney et al., 1968; Mahloudji et al., 1971), teratoma (same site) in sibs (Ewing and Prakash, 1961; Stewart and Bagshaw, 1965; Maganini, 1967; Villani, 1967; Hurlbut et al., 1967), teratoma (different site) over three generations (Lucksch and Ringelhan, 1926), and hepatoma (Fraumeni et al., 1969). In many cases the age of onset was remarkably similar in related patients, especially for congenital or neonatal tumors (medulloblastoma, teratoma, and hepatoma). Numerous cases include more than 2 affected sibs (Roberts and Roberts, 1935; Pohle et al., 1936; Harmon and Morton, 1966) or tumor in more than two generations (Lucksch and Ringelhan, 1926), rendering chance occurrence highly unlikely. Aside from a tissue-specific clustering of familial childhood tumors, studies of childhood cancer (Regelson et al., 1965; Fraumeni and Miller, 1967; Li and Fraumeni, 1969a, 1969b; Miller, 1971) and cancer families (Li and Fraumeni, 1969a, 1969b; Bottomley et al., 1971; Lynch and Krush, 1971) have revealed an association of brain tumors, sarcomas, adrenocortical carcinomas, breast cancer, acute leukemia, and, less frequently, lung cancer, skin cancer, pancreatic cancer, and perhaps other cancers within families and occurring as multiple tumors in the same individual. Numerous familial aggregations of brain tumor, rhabdomyosarcoma and soft-tissue sarcoma, osteogenic sarcoma, and adrenocortical

carcinoma are drawn from such families (Remzi and Kendi, 1966; Kenny et al., 1968; Li and Fraumeni, 1969a, 1969b; Epstein et al., 1970; Bottomley et al., 1971; Miller, 1971). These pedigrees display a pattern consistent with dominant inheritance, an early age of onset for any given tumor, and an earlier age of onset in successive generations. This syndrome may result from an inherited mutation with broad tissue specificity.

There may be more than one inherited mutation which can give rise to a given tumor. For Wilms' tumor (Knudson and Strong, 1972a), two distinct genetic entities were recognized, one occurring alone as the result of a germinal mutation, and one occurring with severe sporadic aniridia, a combination which could result from a deletion involving the same Wilms' tumor. A similar situation may exist for medulloblastoma. As mentioned above, there are reported occurrences in sibs, suggesting the presence of a germinal mutation. Medulloblastoma also occurs in the dominantly inherited nevoid basal cell carcinoma syndrome, with a striking early age of onset and relatively good prognosis (Neblett et al., 1971). The fraction of total medulloblastoma cases contributed by the syndrome cannot be estimated at present, as many patients may die from the medulloblastoma prior to diagnosis of the syndrome. Hopefully more data will be accumulated so that a mutation hypothesis can be tested in the above cases. For now it must be left as a working hypothesis that all childhood tumors fit the two-mutation hypothesis, with a hereditary fraction predisposed by inheritance of a germinal mutation and a nonhereditary fraction not so predisposed. Both types, according to the hypothesis, involve a two-step process. Because it can be inherited the first step is definitely mutational. The second cannot yet be demonstrated to be mutational, although the small mean numbers of tumors in hereditary cases (one to three for the tumors considered) occurring in embryonic tissues that probably number 10^5-10^7 cells suggest an event that is as rare as mutation.

If both events are mutational, do they occur at different genetic loci or at alleles of the same locus? Unfortunately there is no present basis for an opinion in this matter. The allelic hypothesis has been invoked by Nicholls (1969) for the development of *café-au-lait* spots in neurofibromatosis and for the neoplastic lesions found in the phacomatoses generally.

Endocrine

The sympathetic nervous system is the site not only of neuroblastoma, but also of pheochromocytoma. This latter tumor is observed occasionally in childhood, but it is primarily a tumor of young and middle-aged adults, 70% or so of cases being detected between the ages of 20 and 50 years. Generally speaking pheochromocytoma is not malignant, and clinical symptoms result not from mass but from the hypertension caused by the secretion of epinephrine and

norepinephrine. Recent studies suggest that the tumor may be present for some time before it becomes symptomatic.

Pheochromocytoma may be hereditary (dominant autosomal) or nonhereditary, solitary or multiple, and has therefore been subjected to the same kind of analysis of hereditability employed for the childhood tumors (Knudson and Strong, 1972b). It occurs earlier in familial cases (mode, 20 years) than in nonfamilial cases (mode, 35-40 years). In familial cases 44% of patients had bilateral adrenal tumors and 52% had tumors at more than one adrenal or sympathetic nervous system site, whereas only 12% of unselected cases were multiple. These multiple cases are probably of the hereditary types, particularly since the modal age of onset is approximately 20 years, as with familial cases. Familial cases occur earlier and tumors are more often multiple, as observed for childhood tumors. The fact that 39% of childhood cases are multiple is therefore hardly surprising. It is estimated that 22% of cases of pheochromocytoma are of the hereditary type. Penetrance for the carrier state is nearly complete. By the age of 50 years it is at least 90%, and the mean number of tumors occurring in carriers is estimated to be between two and three. The coefficient of selection is difficult to estimate but is calculated to be at least 0.07 (Knudson and Strong, 1972b). From equation (1) a germinal mutation rate of 9×10^{-6} per generation is calculated. This rate falls in the range observed for the three childhood tumors.

It is further concluded (Knudson and Strong, 1972b), as for embryonal tumors, that simple pheochromocytoma results from two events in all cases. The first event is definitely mutational and occurs in germinal cells in 22%, in somatic cells in 78%. The second event, which may be mutational, occurs in somatic cells in all cases.

The hereditary cases noted so far have no associated lesions and are therefore called simple pheochromocytoma. There is a second genetic (autosomal dominant) entity in which the tumor is associated with medullary carcinoma of the thyroid (Knudson and Strong, 1972b). This syndrome accounts for about 30% of all familial cases of pheochromocytoma. Not all patients with the syndrome give a positive family history but they are probably all carriers of the gene which determines the syndrome. The distribution of ages at diagnosis is later (mode 30-35 years) than for hereditary simple pheochromocytoma but is identical for familial and nonfamilial cases of the syndrome. An important clinical difference between simple and syndrome cases is that hypertension in the former is often sustained, whereas in the latter it is virtually always paroxysmal. Tumors in the simple cases are found in both adrenal and extra-adrenal sites and are primarily norepinephrine-secreting, whereas those in the syndrome are found only in the adrenal and are primarily epinephrine-secreting. Bilateral adrenal tumors occur in 75-80% of syndrome cases, whether familial or not.

The medullary carcinoma of the thyroid which accompanies the pheochromocytoma is much more often the cause of death than is the latter tumor. Penetrance of the gene is essentially complete for both tumors by the age of 50 years. A mean number of four tumors is calculated for both the adrenal and the thyroid tumor (Knudson and Strong, 1972b). This calculation lends support to those who believe that all patients with the syndrome should have total thyroidectomy. The coefficient of selection for the syndrome is difficult to estimate. It is at least 0.1, but could be much higher. This value leads to a calculation of a minimal germinal mutation rate of 1×10^{-6} per generation.

The syndrome may be interpreted in the framework of the two-step model too. The first step is a germinal mutation specifically affecting the adrenal and thyroid medullae. A second step occurring in either of these somatic tissues produces tumor. The mean number of times this occurs by the age of 50-60 years is estimated to be four for each tissue. If the first step should occur as a somatic mutation, the overwhelming probability is that only one cell in only one of these tissues would undergo the second step as well and become a tumor cell. Such a sequence would give rise to nonhereditary, solitary pheochromocytoma or medullary thyroid carcinoma. Therefore the syndrome would appear only if the first step were a germinal mutation; all cases of the syndrome must be regarded as hereditary.

A variant of the syndrome, also dominantly inherited, shows multiple associated features, including mucosal neuromas. Several features of this syndrome, especially *café-au-lait* spots, are suggestive of neurofibromatosis, but the two syndromes are distinctly separable. The latter may also be accompanied by pheochromocytoma, but in more nearly 10% of cases rather than nearly 100%, as in the mucosal neuroma syndrome (Knudson and Strong, 1972b).

The endocrine tumors discussed so far occur in just two tissues, one secreting epinephrine and norepinephrine, and the other thyrocalcitonin. Familial incidence has also been observed for other endocrine tumors. The picture is much more complicated for the other tumors, however. As more reports accumulate it becomes an ever-increasing possibility that most of these cases are examples of the same hereditary syndrome, multiple endocrine adenomatosis. Patients with this syndrome may have tumors in one or more of the following endocrine organs: anterior pituitary, parathyroid, thyroid (not medulla), pancreas (islet cells), and adrenal cortex. Bronchial and intestinal carcinoid tumors have also been reported. It is now clear that the Zollinger-Ellison syndrome (peptic ulcer, excessive gastric secretion, and non-insulin-producing islet cell tumors) is a part of the multiple endocrine adenomatosis syndrome. The mutation causing the latter syndrome is dominantly inherited and almost completely penetrant by the age of 20 years (Anderson, 1970). Expression of the gene varies greatly depending on which tumors are found. The pituitary tumors may be nonfunctioning or may be associated with prolactin secretion,

acromegaly, hyperthyroidism, or hyperadrenalism. Hyperplasia and/or tumors may occur in the thyroid, adrenal cortex, parathyroid, or islet cells. The mean number of tumors which appear in the various tissues by a given age has not been calculated, but is probably highest for the parathyroid and pancreatic islets. As with the pheochromocytoma-medullary carcinoma of the thyroid syndrome, the presumption is that tumors result from a two-step process, the first of which is germinal and affects all these tissues and the second of which is somatic and affects one or more of the tissues in a stochastic manner. Nonhereditary, solitary tumors in only one tissue are expected when the first mutation is in somatic tissues. Until the incidence of the multiple endocrine adenomatosis syndrome is established, it will not be possible to estimate the fraction of tumors in a given endocrine tissue which is hereditary. That this fraction could be significant is suggested by the fact that, in one series of 100 cases of hyperparathyroidism, 8 patients had multiple endocrine adenomatosis, the Zollinger-Ellison syndrome, pituitary tumor, or medullary carcinoma of the thyroid (Gonder et al., 1970). It is also necessary to decide whether all familial cases of tumors in these endocrine tissues are examples of the syndrome or whether there are also simple familial tumors, as with pheochromocytoma. For example, some of the families reported and reviewed by Jackson and Boonstra (1967) may represent such instances.

Skin

Cancer of the skin is a frequent form of cancer. It is particularly frequent in geographic areas characterized by high levels of sunlight. Basal cell carcinoma is the most frequent type of skin cancer, followed next by squamous cell carcinoma, and lastly by malignant melanoma and some rarer types. Although the basal cell and squamous cell carcinomas are generally thought to be the consequence of prolonged exposure to sunlight, or therapeutic ionizing radiation and chemicals, heritable forms of each of these cancers are also on record.

The clearest example of an inherited form of basal cell carcinoma is provided by the basal cell nevus or nevoid basal cell carcinoma syndrome (Howell and Anderson, 1972). The principal features of the syndrome include multiple basal cell carcinomas which may develop as early as at 2 or 3 years of age but are generally first diagnosed about the time of puberty; jaw cysts which are also usually multiple with early onset; skeletal anomalies, many of which are congenital and are comprised primarily of bridging of the sella turcica, scoliosis, hemi or block vertebrae, spina bifida, and anomalous ribs; ectopic calcification of the falx cerebri and tentorium cerebelli, as well as in ovarian fibromas, basal cell carcinomas, and jaw cysts; and medulloblastoma. The last tumor was observed in one series in 4 of 22 children with the syndrome and who were under age 15 (Dodd et al., 1972). Six patients have now been described in the literature in whom this tumor developed in association with the syndrome (Neblett et al., 1971).

The inheritance of this disorder is clearly that of an autosomal dominant with a high penetrance of over 95%. Expressivity of the syndrome is more variable among than within families. The frequency of the syndrome is not presently known, except that 13 authenticated cases of the syndrome were ascertained from a review of 1158 records on file at the University of Michigan University Hospital for the 10-year period from 1950-1959 with diagnoses of either basal cell carcinomas, epithelioma adenoides cysticum, multiple benign cystic epithelioma, and/or odontogenic cysts. Five additional cases were ascertained but not verified; the frequency in this hospital population thus ranged from 1.1 to 1.6% (Anderson, 1972a).

The syndrome typifies many of the hereditary forms of cancer where the defective gene has effects on several tissues. Moreover, the tumors that develop are multiple and appear much earlier than do nonhereditary forms. In the syndrome, for example, basal cell carcinomas are usually first diagnosed at about 15 years of age, whereas the solitary, noninherited form of basal cell carcinoma first develops around age 50.

Hereditary forms of basal cell carcinoma not associated with any of the stigmata of the basal cell carcinoma syndrome have also been observed (Howell and Anderson, 1972). No opinion can be expressed at present as to whether these represent minimal expressions of the syndrome, the effect of a different allele at the same locus, or the effect of a different gene at a different locus. Some of these examples may represent the inheritance of a gene or genes resulting in a lack of ability to pigment and thus lessen protection to ultraviolet radiation.

Germinal mutation plays little or no role in squamous cell carcinoma of the skin (or any site for that matter). One condition, however, is frequently confused with squamous cell carcinoma of the skin and will be briefly mentioned, namely, generalized keratoacanthoma. It is also called self-healing squamous cell carcinoma of the Ferguson-Smith type. It is a rare dermatosis in which multiple cutaneous neoplasms (keratoacanthomas) develop on the face, ears, arms, thighs, and legs, and, though histologically identical with squamous cell carcinomas, show a tendency to spontaneous healing after several months' duration. Onset is at about 30 years of age, which is significantly earlier than the average of 50 years for the sporadic form of keratoacanthoma. The majority of cases of multiple keratoacanthoma have a positive family history (Sommerville and Milne, 1950; Charteris, 1951; Ereaux and Schopflocher, 1965), and the transmission pattern of the disease is fully consistent with an autosomal dominant mode of inheritance. It may well be that all cases of generalized keratoacanthoma are the consequence of a germinal mutation.

Cutaneous melanoma is an example of a neoplasm which was long thought not to involve a genetic component, primarily for want of genetic investigation. However, a hereditary variety of the disease is now recognized which differs in

several respects from the nonhereditary type and accounts for 1-6% of cases of melanoma (Anderson, 1971a).

Similar to other neoplasms influenced by a germinal mutation, hereditary melanoma develops at a significantly younger age and frequently at multiple sites compared with the nonfamilial type (Anderson, 1971a). Moreover, patients with the familial type enjoy a significantly higher survival than nonfamilial patients; the survival proportions at 5 years are 0.61 and 0.36, respectively. The tumor type also appears to differ in the two groups, familial patients being characterized by a more localized and superficial type of lesion, and perhaps one that is more amenable to treatment than that in nonfamilial patients.

The genetic mechanism underlying the familial type is apparently complex, but seemingly involves at least one major gene which is autosomally transmitted and manifests some degree of dominance (Anderson, 1971a). This gene may influence melanocytes, their differentiation and localization, and may be phenotypically expressed as junctional or compound nevi or other pigmented lesions. But whether these transform into melanoma seemingly depends on a second event, since malignant transformation of some of these nevi occurs primarily in light-complexioned individuals (Anderson, 1971a).

For two of the major skin cancers, basal cell carcinoma and malignant melanoma, there is a dominantly inherited subgroup, as observed for embryonal and endocrine tumors. At present we do not possess the data on skin cancers necessary to calculate the variables noted for the other tumors. It is apparent, however, that the hereditary skin cancers must require two or more steps for their genesis and that the first step is a mutation, applying that term broadly to indicate chromosomal change.

Breast

It is generally presumed that genetic factors play little or no role in the common types of human tumors, or if a genetic component is suspected, it is usually considered to be polygenic in nature. For example, breast cancer has been the subject of an impressive number of genetic studies, but yet, regardless of the source of patients or controls, the type of data, i.e., morbidity and/or mortality statistics, type of investigative protocol, or type of statistical analysis, these studies have consistently indicated a two- to threefold excess of the tumor in close relatives of patients compared to control data (Clemmesen, 1965; Post, 1966), results which have been interpreted as indicating polygenic inheritance. Twin studies, although theoretically promising, have likewise provided little definitive evidence of genetic utility. Hauge et al. (1968), in a summary of cancer occurrence in 4368 same-sexed twins, recorded concordance of breast cancer in 4 of 23 monozygotic twins and in 6 of 47 dizygotic twins, results which suggest a small genetic effect. These past studies, whether on patients or twins, were based on the premise that breast cancer was a single, homogeneous

disease. If the disease were in fact heterogeneous, as evidence now indicates, the effect would be to diminish or obscure any measure or estimate of a genetic effect. This may be one important reason why past studies have failed to provide unequivocal evidence for the importance of heredity in breast cancer.

Present evidence indicates that breast cancer is comprised of at least two general types, one occurring premenopausally, and one postmenopausally (Lilienfeld, 1963; deWaard et al., 1964; Berndt and Landmann, 1969). Subtypes may also exist. The premenopausal type is characterized by benign breast disease and frequent occurrence of thyroid tumors, and the postmenopausal type by hypertension, obesity, and diabetes. deWaard et al. (1964) have proposed that the premenopausal type is associated with excessive ovarian estrogenic activity, whereas adrenal estrogens play an important role in the postmenopausal type. These two general types have also been recently identified in patients with a family history of this cancer (Anderson, 1971b). Genetic factors were found to have a more important role in one type than the other (Anderson, 1972b).

In patients with premenopausal diagnoses, the frequency of breast cancer in their relatives was 6.7%, which was three times higher than the 2.3% in similar-aged control relatives. No significant increase in frequency was observed for relatives of patients with postmenopausal disease compared with controls. When the disease in the patient was bilateral, the frequency in the relatives increased to 13%, and if both premenopausal and bilateral, the frequency was 17%. The frequency in relatives of patients with unilateral disease, whether pre- or postmenopausal, was about 3.4%, only slightly higher than control values (Anderson, 1972b). The frequency of bilateral breast cancer was 10% in all familial patients, and 15% in those with premenopausal diagnosis. The bilaterality rate in the general breast cancer population is about 3%.

These results suggest that genetic factors play a much more important role in patients with early onset of multiple disease than in patients with late onset of a single tumor. The pedigrees of these patients with early and multiple disease and those reported by other workers (Wood and Darling, 1943; Smith, 1968; Dmytryk, 1971), which refer to similar types of patients (and relatives), point to dominant inheritance. Cady (1970) described bilateral cancer occurring at early ages in three sisters; their mother died at age 36 in childbirth, so whether or not breast cancer might have developed later is not known. Since not all female gene carriers develop breast cancer and since many develop only one tumor, we may presume that two or more steps are required and that the first is a mutation which may be germinal (hereditary form) or somatic (nonhereditary form).

If breast cancer occurs in both nonhereditary and hereditary forms and if the hypotheses involved in the two-step model are applied, what would be the anticipated fraction of hereditary cases and what would be the expected concordance rates for monozygotic and dizygotic twins? One hypothesis is that all bilateral cases are hereditary. If the bilaterality rate is generally 3% and for

familial cases 10%, then 30% of all cases should be of the hereditary type. Of course, 30% would not be expected to give a family history because transmission could be through the male line, because penetrance is not complete, and because some carriers have not yet contracted breast cancer or have died of other disease before doing so. Anderson (1971b) has observed that some 8% of 6550 patients with breast cancer gave a history of the disease in grandmother, mother, aunt, or sister. The expected concordance in twins may then be estimated roughly. An identical twin of an affected individual should carry a dominant germinal mutation for breast cancer in 30% of cases, whereas a fraternal twin should carry it in one-half that many, assuming that new mutations play a negligible role in such a common disorder. In addition, either an identical or a fraternal cotwin could acquire the disease on a nonhereditary basis with a probability of 2-3%. The real expectations would be lower than the sums of 0.33 and 0.18 for monozygotic and dizygotic twins, respectively, depending on gene penetrance, but it is interesting that the pooled concordances observed from past twin studies (Macklin, 1940; Harvald and Hauge, 1956; Jarvik and Falek, 1962; Hauge et al., 1968) were 0.28 and 0.14, respectively. If the pairs reported by Harvald and Hauge (1956) are removed, since they may also be included in the series of Hauge et al. (1968), the pooled concordances were 0.28 and 0.12, respectively.

In addition, another hereditary subtype of breast cancer may well be included within the early and multiple group. This type also seems to conform to a dominant inheritance pattern but is characterized by the occurrence in close relatives not only of early and multiple breast cancer, but also of leukemia, brain tumor, sarcoma, and/or carcinomas of the lung, pancreas, and skin (Li and Fraumeni, 1969a, 1969b). Bottomley et al. (1971) recently described a large pedigree which included 37 members with one or more of these tumors, the most frequent being cancer of the breast, sarcoma, and acute leukemia. These tumors were confined to specific branches of the pedigree and appeared to conform to an autosomal dominant mode of inheritance.

Since sarcomas and brain tumors constitute an important fraction of the tumors developing in recipients of renal homografts whose immune mechanisms have been suppressed, this raises the intriguing possibility of a loss or impairment of a surveillance mechanism for neoplastic mutant cells, and/or one permitting the proliferation of oncogenic viruses (Schneck and Penn, 1971) in this type of breast cancer.

Lynch and Krush (1971) recently described cancer of the ovary in 3 of 34 families with cancer of the breast. Both neoplasms were inherited in a dominant fashion and occurred concomitantly in 2 of the index patients. These families also manifested sarcoma and cancer of the lung, colon, and skin. They could, therefore, belong to the subgroup of breast cancer families reported by Li and Fraumeni (1969a, 1969b) and Bottomley et al. (1971), but the data are still too meager to evaluate this or other possibilities.

Digestive Tract

Cancer of the large intestine is a frequently occurring neoplasm and is second only to lung cancer as a cause of death in the United States, according to American Cancer Society statistics for 1971. But unlike lung cancer, cancer of the colon (and rectum) is clearly divisible into several distinct heritable forms (see review by McConnell, 1966). The most common of these is familial polyposis (profuse carpeting of the entire colon and rectum by polyps). If untreated, most patients will almost invariably die from carcinoma of the colon before age 50 (McConnell, 1966). Onset of symptoms averages about 30 years of age, age at diagnosis about 33 years, and age at death about 40 years (Veale, 1965), which is significantly younger than the 68 years of age at death from carcinoma of the colon in the general population. The disorder is transmitted as an autosomal dominant trait (Veale, 1965).

Familial polyposis coli is one of the few genetic disorders associated with malignancy for which estimates of the population frequency, relative fitness, and mutation rate are available. The population frequency of the disorder has been estimated in three studies. Reed and Neel (1955) provided an estimate of 1 in 8300, which they considered an underestimate. Veale (1965), using statistics from St. Mark's Hospital in London, arrived at an estimate of 1 in 23,790. A more recent estimate, by Pierce (1968), based on a Kentucky survey, was of the order 1 in 6850. In spite of these differences, the relative fitness of individuals with the gene was highly similar, 0.78 in the study of Reed and Neel (1955), 0.82 in the study of Veale (1965), and 0.85 in the study of Pierce (1968). The mutation rates in these studies ranged from 12 to 38×10^{-6} loci per generation.

Gardner's syndrome is another genetic entity characterized primarily by multiple polyps of the colon and occasionally of the small intestine; sebaceous and epidermoid cysts, fibromas, and desmoids; and osteomas of the facial bones (Pastinszky et al., 1969). The liability to colon cancer in this disorder is high. The onset of symptoms and age at diagnosis of polyps and cancer are similar to those in familial polyposis (Pierce et al., 1970). The numerous published pedigrees of the syndrome clearly indicate an autosomal dominant mode of inheritance and a high degree of penetrance of the mutant gene (Pierce et al., 1970). No clues concerning the nature of the gene effect that causes the diverse stigmata of the syndrome are yet available. Patients with the syndrome, however, are refractory to the phosphaturic effect of parathyroid extract on the renal tubules and possibly the bones (Trygstad et al., 1968). The frequency of Gardner's syndrome has been estimated at 1 in 14,025, the relative fitness at 0.82, and the mutation rate at 13×10^{-6} loci per generation, according to a recent study by Pierce et al. (1970).

Peutz-Jegher's syndrome is a relatively infrequent genetic entity in which melanin pigmentation of the oral musoca, lips, face, fingers, and toes is

associated with polyps, primarily of the small intestine but possibly also of any region of the gastrointestinal tract (Jeghers et al., 1949). The polyps are considered to differ from those in the other polypoid diseases in that they are hamartomas and not adenomas, and not considered to be precancerous. However, several patients have now been described with a primary gastrointestinal carcinoma, presumably arising from preexisting polyps (Morson, 1962; Williams and Knudsen, 1965). Thus, it appears that Peutz-Jegher's syndrome is a precancerous condition, but with a smaller risk of cancerous transformation than familial polyposis coli or Gardner's syndrome. The syndrome follows a dominant inheritance pattern (McConnell, 1966).

Evidence is beginning to accumulate showing impressive aggregations of colon cancer in successive generations of families which manifest no evidence of either a preexisting precancerous condition or an associated nonneoplastic condition. In addition to adenocarcinoma of the colon, these families are of further interest because adenocarcinomas may also develop in the uterus, stomach, and occasionally the ovaries. Another important characteristic is that the adenocarcinomas of the colon are generally first detected at the relatively early age of 40 years, which is one or two decades earlier than the nonhereditary type of colon cancer (Anderson, 1970). A sufficient number of patients and families with these associated and early-occurring adenocarcinomas have now been observed to indicate that they constitute a distinct disease entity, which is referred to as hereditary adenocarcinomatosis. It is inherited in a dominant fashion, with a penetrance of 90% in a carrier of the mutant gene who is at least 25 years of age (Anderson, 1970).

A heritable component is also involved in some cases of stomach cancer which develop independently of colonic polyps or cancer. A number of retrospective genetic studies have indicated that stomach cancer is two to four times more frequent in first-degree relatives of stomach cancer patients than in control relatives (McConnell, 1966). The hereditary tendency in these studies may reflect, at least in part, the association that exists between stomach cancer and blood type A, since individuals with this blood type are about 20% more liable to the occurrence of stomach cancer than those with blood groups O, B, and AB (McConnell, 1966). Several pedigrees have also been reported in which stomach cancer occurs in direct descent through several generations, suggesting that this neoplasm may also be comprised of one or more dominantly inherited types (Macklin, 1960b; Cruz et al., 1961; Woolf and Isaacson, 1961; Kopf et al., 1967).

Only one report was found pointing to a familial occurrence of esophageal cancer (Freytes and Carri, 1968), but several families have now been described in which carcinoma of the esophagus occurs in association with tylosis, also called keratosis palmaris et plantaris (Howel-Evans et al., 1958; Shine and Allison, 1966; Harper et al., 1970). The skin disorder is characterized by thickening and

fissuring of the palms and soles; onset is usually between 5 and 15 years of age. Another form of tylosis, occurring even earlier in life, is not associated with esophageal cancer. Both forms of tylosis are inherited in a dominant fashion. Howel-Evans et al. (1958) described two families with tylosis occurring between 5 and 15 years of age. Of special interest was the finding that of 48 tylotic family members, 18 developed esophageal carcinoma at an average age of 45 years. It was estimated that 95% of those with tylosis would develop the neoplasm if they survived to 65 years of age. No cases of esophageal cancer developed in family members who were free of tylosis. Another family with this unusual association of disorders has also been reported recently (Shine and Allison, 1966). However, this family differed from the previous one in that a congenitally abnormal esophagus was observed in two or perhaps three generations. The esophageal cancer occurred at an average age of 61. The findings from this latter family have been interpreted as indicating a different allele from that in the families reported by Howel-Evans et al. (1958), which in turn was considered different from that usually responsible for the diffuse form of tylosis. Observations on the two original families by Howell-Evans et al. (1958) were recently updated by Harper et al. (1970), who also described two additional families.

For the common cancers of the digestive tract it is apparent that there exists at least one dominantly inherited form. Again we may conclude that these cancers can arise as a result of two or more steps, the first of which can be a germinal mutation.

Genitourinary

Germinal mutation plays an important role in cancer of the ovary. These genetic types may arise from surface epithelial cells, germ cells, or sex cord mesenchyme. The poorly differentiated embryonal tumors, referred to as gonadoblastomas or dysgerminoma, are frequently found in patients with XY gonadal dysgenesis and in the testicular feminization syndrome, both of which are male-limited disorders and could be inherited either in an autosomal, sex-limited dominant or X-linked recessive fashion. There is little evidence that the testicular feminization syndrome is closely linked to any acknowledged sex-linked condition, suggesting that the autosomal mechanism is most likely (Hamerton, 1971). Gonadoblastomas or dysgerminomas, often bilateral, develop in 30-40% of patients with dysgenic gonads (Hamerton, 1971; Talerman, 1971). The mutation rate of this syndrome has been estimated at 4×10^{-6} loci per generation if autosomal and 5×10^{-6} if sex-linked (Hamerton, 1971).

Dysgerminomas in three generations of a family and occurring independently of XY gonadal dysgenesis or the testicular feminization syndrome were reported by Jackson (1967). Of the two documented cases in this family, a mother developed mixed chorionepithelioma and dysgerminoma at age 12. Dysgermino-

mas as well as sex cord tumors have been reported in about 5% of patients with the dominantly inherited Peutz-Jeghers syndrome (Scully, 1970b).

Arrhenoblastoma is an infrequent, masculinizing type of ovarian tumor. Its occurrence in first cousins, sisters, and a mother and daughter has been described (for references see Goldstein and Lamb, 1970).

Papillary cystadenocarcinoma has now been observed in three well-documented families (Li et al., 1970). The distribution pattern of the tumor is fully compatible with a dominant mode of inheritance with a penetrance value over 90%. Similar to other hereditary tumors, papillary cystadenocarcinoma occurs earlier than ovarian cancer in general, but no information is presently available on the frequency of bilaterality. Papillary adenocarcinoma has been reported to have a bilaterality rate of 51% (Soini and Serra, 1958), but whether this estimate was based on patients with the heritable or nonheritable types, or both types, is not known.

Ovarian cysts and fibromas are a frequent finding in the nevoid basal cell carcinoma syndrome, and ovarian adenocarcinomas have been observed in 2 of 22 adult females with the syndrome (Howell and Anderson, 1972; Anderson, 1970). Ovarian adenocarcinomas may also occur with hereditary adenocarcinomatosis (Anderson, 1970).

Tumors of the normal testis are much rarer than tumors of the normal ovary. Diagnoses center about three diagnostic categories, seminoma, teratoma, and embryonal carcinoma, although elements of more than one type may be found in a single tumor, all being derived from the germ cell line. Several instances of concordance have been recorded in definitely monozygous twins (Champlin, 1930; Salm and Adlington, 1962; Stewart and Bagshaw, 1965; Villani, 1967) and in nontwin brothers (Raven, 1934; Lownes and Leberman, 1939). These familial cases include all the above tumor types.

A major genitourinary cancer site is the uterus, which is actually two sites. There is very little evidence to suggest that germinal mutation plays a significant role in carcinoma of the cervix, and that disease will not be discussed (Rotkin, 1966). Quite the opposite is true for carcinoma of the corpus uteri, however. In a series of 154 consecutive patients with this diagnosis Lynch et al. (1966) found 20 with a history of endometrial carcinoma in a first-degree relative. Of the 20 mothers of these probands 12 gave a history of endometrial carcinoma. There were also 11 affected sisters. These pedigrees are quite compatible with highly penetrant dominant inheritance and provide a minimum estimate of 13% of cases occurring in genetically predisposed individuals.

These familial cases of endometrial carcinoma were found to have primary malignant tumors at other sites in 11% of cases (Lynch et al., 1966), but the sites were not consistent. On the other hand, the cases of hereditary adenocarcinomatosis noted earlier, in which carcinomas of the colon and endometrium have been found in the same large pedigrees, are consistent with a

dominant gene affecting primarily those two tissues (Anderson, 1970). This is apparently a separate mutation from the one just described.

Endometrial carcinoma has also been associated with a triad of obesity, hypertension, and diabetes mellitus (Baanders-van Halewijn et al., 1964; Lynch et al., 1966), similar to the association with cancer of the breast. There is no evidence that this association is determined genetically, although the genetic subgroup reported by Lynch et al. (1966) displays the entities of the triad with approximately the same frequencies as do the cases with a negative family history.

In at least one instance an endocrine dyscrasia does predispose to endometrial carcinoma. In the case of the Stein-Levinthal syndrome, the characteristic findings of amenorrhea, sterility, hirsutism, obesity, and cystic ovaries are frequently (38%) (Jackson and Dockerty, 1957) accompanied by endometrial carcinoma. The Stein-Levinthal syndrome itself may be a dominantly inherited disorder (Cooper et al., 1968).

In males the most common genitourinary cancer, carcinoma of the prostate, has been reported frequently in more than one member of a family. For example, Woolf (1960) found that deaths from prostate cancer were three times as frequent among male relatives of propositi as among controls. Woolf concludes that an organ-specific genetic component is present in prostatic cancer.

Finally, mention can be made of four families (Rusche, 1953; Brinton, 1960; Klinger, 1968; Griffin et al., 1967) in which hypernephroma was found in more than one member. In two of these more than one generation is involved and dominant inheritance is suggested. Similarly there have been several reports of bladder cancer in more than one member of a family. In one of these (Fraumeni and Thomas, 1967) a father and 3 sons were affected.

Unfortunately there are not enough data to analyze all the genitourinary cancers in detail. It does appear that all could have a dominantly inherited subgroup or subgroups, and this is clearest for ovarian and endometrial cancers.

Lung

Bronchogenic carcinoma is truly a twentieth-century disease. At the beginning of the century it was a relatively rare disease, and during one lifetime it has become the greatest cause of cancer death in the United States. Of all the common cancers it is the one most clearly associated with environmental agents. Yet there is definite evidence for familial clustering which can be dissociated from environmental factors. Tokuhata (1964) has shown that both heredity and cigarette smoking are important; individuals who have an affected close relative and who smoke have a 14-fold increase in risk of lung cancer, greater than that associated with either factor alone. The nature of any genetic factor is unknown.

Perhaps future reports of pedigrees of multiple cases will be helpful. Two recent reports are those of Brisman et al. (1967) on 3 brothers and a sister, and of Nagy (1968) on 3 brothers, with lung cancer. In addition carcinoma of the lung has been found in association with the dominantly inherited Hamman-Rich syndrome of diffuse interstitial fibrosis of the lung (Swaye et al., 1969). Although most lung cancer probably does not occur in individuals predisposed by a dominant mutation, the latter may well exist and in so doing demonstrate that lung cancer too can occur in man as a result of two or more steps, the first of which is a mutation.

Leukemia, Lymphoma, and Multiple Myeloma

The increased incidence of leukemia and lymphoreticular neoplasms in various genetic disorders has already been discussed. Those findings bear only peripherally on the question of a dominantly inherited form of any of these neoplasms. Here we shall inquire what fraction of these diseases, if any, might occur in dominant gene carriers.

Data bearing on this question come from twin studies and from family studies. Twin studies indicate a distinct hereditary factor(s) in leukemia; family studies suggest there is at least one dominantly inherited factor in leukemia.

Until the report of MacMahon and Levy (1964), reports on leukemia were not based on any large-scale survey and were very possibly biased toward excessive concordance. These authors state that over 20 of the reported concordant twin pairs were monozygotic and that none of the 3 concordant dizygotic twin pairs is well enough documented to be accepted. These reports do establish that the incidence of concordance in monozygotic twins far exceeds random expectation. MacMahon and Levy analyzed 52 consecutive twin sets selected for the presence of one affected member. Of these sets 22 were estimated to be monozygotic. The second twin was also affected in 5 instances, all monozygotic. The authors conclude that concordance for leukemia is of the order of 25%. In this survey all the concordant cases were diagnosed as acute leukemia in childhood. This accords with previous literature, except that chronic lymphatic leukemia, but not chronic myeloid leukemia, has been reported as concordant in a number of instances.

The findings of MacMahon and Levy seem to be confirmed by a more recent report by Miller (1968). In a survey of death certificates over a 5-year period Miller found leukemia as a cause of death in both members of 5 pairs of like-sex twins. The type of leukemia was not specified for 8 of the 10 cases. The concordance estimated by MacMahon predicted 9 affected monozygotic pairs, but Miller points out that the short period of his own study tends to cause an underestimate. Miller views his finding as a confirmation of the earlier report.

Two features of the concordant twin reports are remarkable. One is that the

ages at diagnosis of concordant affected twins are earlier than for childhood leukemia generally (Pearson et al., 1963). Leukemia is like familial cancers in general in this regard. A second feature is that the interval between onset in the two members of an affected sib pair is often brief. Since genetic factors held in common monozygotic twins could affect time of occurrence of a somatic mutation or other somatic event which follows as inherited mutation, this finding does not rule out a two-step mechanism. However, the finding has raised the question whether one of the children might have received transformed cells into his circulation from his cotwin in utero (Wolman, 1962; MacMahon and Levy, 1964; Clarkson and Boyse, 1971). This hypothesis could be tested by the use of a cytogenic or X-linked enzyme marker as employed to test the single-cell origin of cancer. One report which contradicts the hypothesis relates the finding of a marker chromosome in some cells of one of a monozygotic twin pair, but not in the other (Pearson et al., 1963). Unfortunately this case cannot be regarded as conclusive because both patients had been treated and were studied in relapse. Of course such a hypothesis could not explain the high concordance rate noted for retinoblastoma and perhaps other childhood tumors. On the other hand, the two-step hypothesis predicts a concordance rate in the range of 0.14-0.38 for three childhood tumors, as noted above, according to the expression $f_h(1 - e^{-m})$. If there is a similar fraction of childhood leukemia caused by a new dominant mutation, or by inheritance of a previous mutation, a value of 0.25 for concordance would be quite in keeping with the other cancers.

Numerous instances of 2 or more familial cases, not twins, of leukemia have also been reported. Most instances are in sibs, although the risk to a sib of an affected individual has not been measured precisely. The highest estimate of familial incidence has been that of Videbaek (1958) of 8% in Denmark, with all estimates in the United States being less than 1%; in fact Steinberg (1960) found the familial incidence of leukemia to be no greater than that expected by chance. It may be that more detailed family histories would reveal a higher familial incidence, a conclusion favored by the report of Rigby et al. (1968) in Nebraska, who found 112 probands with no family history of leukemia or lymphoma and 39 probands with such a history. These 39 families had 91 of 855 total family members affected by acute leukemia (32), chronic lymphocytic leukemia (20), Hodgkin's disease (15), lymphoma (19), or multiple myeloma (5). These 91 individuals ranged in age from infancy to 80 years, with a strong shift toward younger ages than those encountered for the 112 probands with no family history.

The reported patterns of inheritance of leukemia are very similar to those noted for the childhood tumors. In many instances sibs have been affected with acute leukemia during childhood, and there is no further familial incidence. Gunz et al. (1966), in reporting a new sibship of 4, possibly 5, affected members, have referred to the earlier literature of instances of 3 or more affected sibs.

Several recent reports of this type have been made (Lundmark et al., 1967; Fraumeni et al., 1969; Potolsky et al., 1971). In these reports of sibs with leukemia (and lymphoma) the diagnostic categories have not included chronic myeloid leukemia, except for the previously mentioned families of Tokuhata et al. (1968) and Hirschhorn (1968). In one report of chronic myelocytic leukemia in infant siblings (Holton and Johnson, 1968) the hematologic findings included thrombocytopenia, a feature typical of the infantile Ph^1-negative disease (absence of Philadelphia chromosome). In the experience of one of us (A. G. K.) this disease was identified in 1 of 2 leukemic first cousins, the other of whom had acute lymphatic leukemia. The child with "chronic myeloid leukemia" had Ph^1-negative cells (Reisman and Trujillo, 1963, case 7; Knudson, 1965). This form of chronic myeloid leukemia should be considered a separate entity from the usual Ph^1-positive form, which has not been found in any of these pedigrees.

From the reports in twins and sibs no Mendelian pattern can be discerned. However, there are also numerous reports of vertical transmission consistent with irregular dominant inheritance much like that seen in retinoblastoma. The pedigrees reported by McPhedran et al. (1969) show this particularly well. In one branch of this pedigree 2 unaffected sibs each had 3 offspring with chronic lymphatic leukemia. In a branch of the pedigree related by marriage, there were 4 individuals with acute leukemia among three generations of descendants of 2 sisters. In the family reported by Heath and Maloney (1965) 5 cases of acute leukemia were found in members of three generations: a woman, 2 of her sibs, her daughter, and her grandson. The family of Snyder et al. (1970) contained 6 cases of acute myeloid leukemia and 2 cases of malignant reticuloendotheliosis over three generations. Four cases of acute leukemia in four generations of a family were recorded by Ferguson and Lynn (1970).

Some features of leukemia and lymphoma resemble those of dominant forms of embryonal tumors. There is a high concordance in identical twins in childhood, affected sibs are too frequent to be explained by chance, transmission from generation to generation has been observed a number of times, and familial cases develop the disease earlier than do nonfamilial cases. With leukemia separate "primaries" cannot be counted as with solid tumors, although the genetic hypothesis predicts such instances. Use of X-linked markers could be very helpful in this respect, although there is now the possibility that cells may "acquire" leukemia from the host, as happened to cells transplanted into a patient with acute lymphoblastic leukemia (Fialkow et al., 1971). The available evidence does point strongly toward a dominantly inherited subgroup of leukemia and lymphoma. Whether this subgroup accounts also for instances in sibs and twins cannot be decided now. Therefore, the total contribution, i.e., the hereditary fraction, f_h, cannot be estimated at this time. If it develops, however,

that concordance of childhood leukemia in monozygotic twins is attributable to a dominant gene, this fraction would be of the order of magnitude of 0.25.

Associated with leukemia and lymphoma in some pedigrees is multiple myeloma, a neoplastic disease of plasma cells. Multiple myeloma itself has been observed a number of times in close relatives. Unfortunately there are not as good data on it as on other entities in this category. Yet it offers an unusual opportunity. It is the only cancer whose genetic expression at the molecular level can be detected. The neoplastic cells in this disease synthesize a monotonous immunoglobulin which varies from case to case. If cancers generally arise by a mechanism which entails two or more steps, and if the first step is associated with tissue specificity, then sibs should have neoplastic clones which resemble each other with respect to gene-determined antigenic specificity but which differ from each other with respect to the second step. Although there are not yet enough critical data on this question, two instances of myeloma in relatives (sister-sister and mother-son) are of considerable interest. In both instances the light-chain specificities (kappa or lambda) were the same for the two relatives but the patterns of serum M-components and/or Bence-Jones proteinuria were different (Leoncini and Korngold, 1964; Berlin et al., 1968). This finding accords with expectation. Obviously more cases need to be studied.

A dominantly inherited form of multiple myeloma should show other features of hereditary tumors: tumors should occur earlier and they should sometimes be multiple. This latter prediction is especially interesting, because it can be tested. Since each case of multiple myeloma is characterized by a unique globulin-producing cell line, the presence of two different myeloma globulins would indicate two separate clones of tumor cells. So far, however, there is only one report of transmission of multiple myeloma from one generation to the next (Nadeau et al., 1956).

GENERALIZATIONS CONCERNING HEREDITARY TUMORS

In view of the foregoing review of hereditary tumors it becomes a reasonable deduction that for each class of cancer there is one or more dominantly inherited form. In some instances the responsible gene specifies more than one kind of tumor, but even when it specifies several, as in the case of hereditary endocrine adenomatosis, the range of phenotypic expression is limited.

This heritable cancer mutation is not sufficient to initiate a tumor; if it were, the expressing cells would each give rise to tumor. The simplest hypothesis is that a second event must occur before a cancer cell is formed. Nothing is known about the nature of this event but one possibility is that it too is a mutation. A simple two-step process probably operates for at least some tumors, notably those of childhood, although it is possible that others develop in more than two steps.

Nonhereditary cancers are visualized as resulting from a two-step process too. The second step is presumed to be identical with that in hereditary forms of the same tumor, occurring in somatic cells in both instances. The first step is regarded as a mutation in both hereditary and nonhereditary forms, germinal in the former and somatic in the latter.

Those who carry a germinal mutation have cells in a specified tissue which are all one step toward being cancer cells. Such carriers are therefore far more likely to develop tumors than are noncarriers. They will also be expected to get them earlier on the average. Then too, they may develop more than one tumor in a specified tissue, and, in the case of paired organs, may acquire bilateral tumors.

Until recent times children with cancer have usually not survived, so that such germinal cases could not reveal the dominant mutation they carried. In effect such mutations have been dominant lethals. Now there are survivors of these tumors, and we may anticipate that offspring of these survivors will sometimes develop tumors too; in fact, this has happened many times with retinoblastoma. The implication for genetic counseling is apparent, but means are needed for identifying which isolated cases of a tumor are new germinal mutations.

There is great specificity in the dominant cancer mutations, even when more than one tissue is specified. This suggests that the first (mutational) step involves a tissue-specific gene, such as one which is expressed at a cell surface.

IMPLICATIONS OF A GENETIC MODEL FOR CARCINOGENESIS

Whether some molecular event is common to the origin of all cancers is unknown at this time. If that were the case, however, any genetic hypothesis for carcinogenesis would need to be reconciled with the body of knowledge available on environmental carcinogenesis. Before embarking upon a discussion of this reconciliation, let us consider a minimum genetic hypothesis that would account for the previous observations on human cancer.

The existence of both hereditary and nonhereditary forms of tumor has been accounted for above by a two-step model. Since the first step can be inherited, it is called mutational, although that designation is not used for any specific kind of genomic change and could encompass such entities as point mutation, frameshift mutation, deletion, duplication, rearrangement, or the addition of a viral genome. The second step might be mutational but there is no compelling evidence for that conclusion. These two steps constitute the minimum model, and further steps might be necessary for the origin of some tumors.

What is the evidence from research on environmental carcinogenesis that two or more steps are necessary? Actually the first clear evidence for a two-step model came from experimental work on chemical carcinogenesis. Berenblum (1941) used two different kinds of carcinogens and was able to demonstrate that the sequence in which they were applied was critical. Benzpyrene and croton

oil caused much more skin cancer in mice when applied in that sequence than in the reverse sequence. Friedewald and Rous (1944), observing that skin tumors induced in rabbits by polycyclic hydrocarbons regress spontaneously but can be made to reappear, concluded that tumor production involved two steps, one initiating, the other promoting. The hydrocarbon was referred to as an initiator and croton oil as a promoter. Promotion involves stimulation of cell proliferation, whereas initiation has been viewed as change of another kind.

Another kind of carcinogen is the tumor virus. The prototype virus for in vitro transformation has been polyoma. Hamster embryo cells are initially converted by it into thin clones of cells which are refringent and randomly oriented. From these clones arise thick, multiple-layered clones of typical tumor cells. Transformation is conceived as a two-step process, both of which steps are affected by virus (Vogt and Dulbecco, 1963).

For still another carcinogen, radiation, there is an a priori consideration that the dose-response relationship could yield information which discriminates between a one-hit and a two-hit mechanism. Thus, for example, single chromosome breaks (one hit) show a linear dose-response curve, whereas rearrangements (two or more hits) show an exponential dose-response curve. Unfortunately, both types of response are conceivable, depending on whether radiation affects one or both steps. Studies on radiation leukemogenesis favor the linear, one-hit type of dose-response curve (Lewis, 1970).

Another kind of evidence which is related to these examples is the body of data on the relation between incidence of various cancers and age of onset. If we assume that the number of cells at risk and the risk per cell are both constant throughout a lifetime, then the accumulated incidence of a cancer will vary linearly or exponentially with time depending on whether one hit or more than one hit is required. In fact, the accumulated incidence of all adult cancers increases exponentially with age. The chief question asked by epidemiologists has been whether the curves favor a two-hit or a multihit hypothesis (Ashley, 1969). These estimates are plagued by lack of quantitative data on the age dependence of such parameters as number of target cells, efficiency of DNA repair, and effectiveness of immune defense against cancer cells.

These pieces of evidence by no means prove that carcinogenesis always occurs in a two-or-more-step process, but they do render any one-step hypothesis unpromising. More important is knowing whether the process involves two or more than two steps. Unfortunately there are too many variables which cannot be measured to be able to decide that matter with any confidence, although estimates as high as seven steps have been made for some cancers (Ashley, 1969).

The mutation model not only calls for two or more steps but also requires that at least the first step be mutational. This is not inconsistent with observations on environmental carcinogens. It is too well known to repeat here that the agents noted above interact with host cell DNA and therefore could be

exerting their effects by genomic change, or mutation, broadly speaking. Still unsettled is the question whether effects on DNA are primary or whether effects on RNA, proteins, or other molecular species are primary.

A mutational mechanism is most convincing in the case of radiation carcinogenesis. A critical contribution has come from the human disease, xeroderma pigmentosum, already cited above. The fact that repair of ultraviolet-induced damage to DNA is defective is direct evidence that radiation can produce cancer via mutation.

Also supporting the notion that radiation acts via mutation is the observation that not only are the offspring of mothers irradiated to the abdomen during pregnancy at increased risk for leukemia but so also are the offspring of mothers irradiated prior to conception (Graham et al., 1966). This finding lends support additionally to the hypothesis that some leukemia is prezygotic.

Unfortunately we do not have information of this kind for chemical carcinogens or for tumor viruses. There is of course the abundant evidence that tumor viruses can be transmitted vertically, but this is not necessarily associated with integration into the host genome. Furthermore, allelic change at the H-2 histocompatibility locus in mice can determine susceptibility to leukemogenesis by leukemia viruses (see, for example, Lilly, 1970). Studies on chemical carcinogens increasingly suggest that initiators act via mutagenesis and promoters act via stimulation of cell division (Ryser, 1971).

The hereditary tumors leave an important implication for experimental studies. None of the hereditary syndromes is without tissue specificity. In some instances, as in hereditary adenocarcinomatosis, that specificity is for several major cancers, but there are no examples of general predisposition to cancer. Since the inherited change is a mutation and since it represents the first of a small number (perhaps two) of steps, it could be very revealing to know its physiological effect. The tissue specificity the hereditary tumors display draws attention to known agents of expression of such specificity, particularly to cell membrane macromolecules, one of whose roles is to provide the cell-to-cell recognition thought to be defective in cancer (Carter, 1968).

Finally, there are two important implications if all human cancers occur in at least one hereditary as well as nonhereditary form:

(1) Different distributions of the germinal mutations in different populations would lead to ethnic differences in incidences of specific tumors.

(2) Any physiological manifestation of the inherited mutation could be used to identify individuals at high risk.

CONCLUSIONS

Two categories of genetic disease are associated with cancer in man. One category includes a group of chromosomal and Mendelian disorders which are

associated with increased, but by no means invariant, risk. The other includes highly penetrant hereditary (autosomal dominant) tumors.

The first group of disorders informs us that chromosomal aberration may still be a primary event in carcinogenesis. It also points to the immune mechanism as an important parameter in the development of at least some cancers.

Virtually every cancer which occurs in man occurs in a genetic (autosomal dominant) form as well as in a nongenetic form. In the genetic forms, at least one more change must occur before tumor develops; tumor develops after a total of two or more changes. Nongenetic forms probably also result from two or more changes, but both occur in somatic tissues. In all forms of cancer two or more changes are necessary. The first change is apparently mutational, using that term broadly to designate heritable chromosomal change. The mutation is specific for one or more tissues. Further changes may be mutational as well, but other possibilities are not eliminated. Genetic forms of tumors occur earlier than do nongenetic forms and are frequently multiple. In theory the gene carriers could be identified as predisposed individuals, and in fact can be identified in some selected cases.

Data on environmental carcinogenesis are consistent with a two-step model in which at least the first step is mutational.

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CHAPTER 4

On the Application of Knowledge to the Patient with Genetic Disease

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At one time the practice of medicine was dominated by patients with acute illnesses of infectious and environmental origin. Progress in medicine has reduced the number of such patients, and the profile of major illness has changed. Patients with degenerative disease such as strokes, heart disease, and cancer fill our general hospitals and dominate the attention of the policy makers who decide, as best they can, how to spend the health resource budget. Many see an opportunity to anticipate the appearance of later illness by early diagnosis in the pediatric age group. Those interested, for example, in coronary artery disease want a foothold in pediatric medicine, hoping by manipulation of food habits early in life to change the incidence of heart disease in later life (Report of Inter-Society Commission, 1970). Although there are many questions to be answered about the wisdom of that approach in this particular example, it should be possible to isolate at least one important genetic determinant of coronary artery disease and to study the effect of environmental control on its natural expression. The obvious example is type II hyperlipoproteinemia (Frederickson and Lees, 1966), and there is hope that this hereditary disease could become an important model for the long-term study of multifactorial heart disease (Frederickson, 1971). From such beginnings perhaps we might learn how to approach the control of other multifactorial diseases.

In this article, we would like to describe the way in which a genetics center in general may, and one integrated program in particular does, carry out its role in helping the patient with hereditary illness. As the opening comment indicates, what is genetic and what is nongenetic is not always clear, and the boundary line is likely to change as our knowledge of disease improves. McKusick (1971a, b) has described, in numerical terms, the growth of our knowledge about identifiable genetic entities; Fig. 1 summarizes McKusick's tabulation of the number of

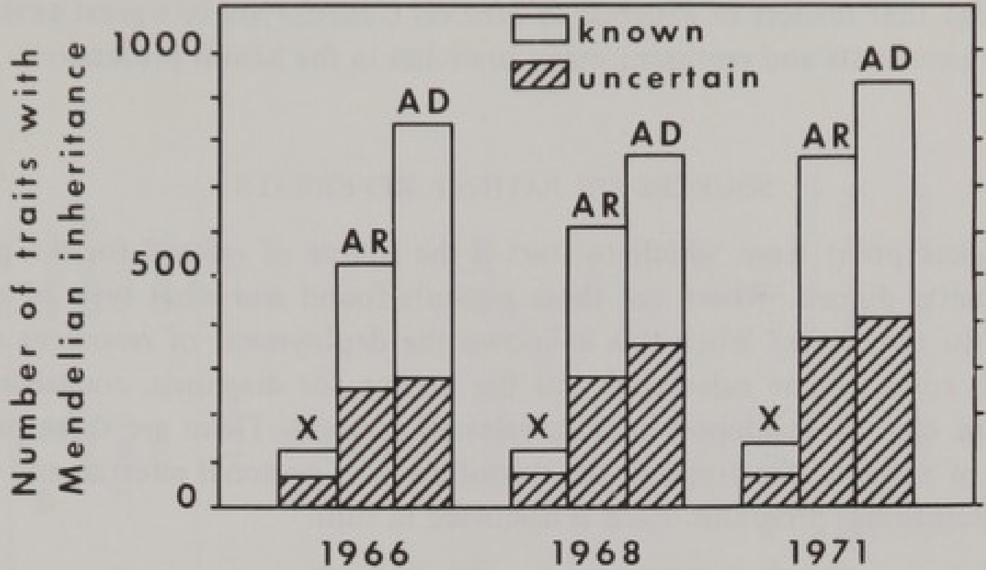

FIG. 1.–Known and probable diseases of man exhibiting Mendelian inheritance. Chart indicates increase in number of diseases classified as genetic and recorded in McKusick's catalogue of Mendelian traits. (AD, autosomal dominant; AR autosomal recessive; X; X-linked.) Adapted from McKusick (1971a, 1971b).

Mendelian traits which the physician can classify. The variation from one edition to the next of McKusick's catalogue does not necessarily reflect any change in the *occurrence* of genetic disease but rather change in the *recognition* and *classification* of genetic disease; this aspect of genetic nosology will no doubt continue for a long time to come. Physicians and their geneticist colleagues have become more skilled in sensing variation in expressivity of certain mutant genes and the genetic heterogeneity underlying other hereditary traits. This awareness has led to refinement in classification, an advance which can only help the patient with genetic disease and those responsible for his diagnosis, counseling, and treatment.

We have chosen to present this discussion under five headings and without an exhaustive review of the literature. In the first section (source of patients), we examine the genetic disease burden in our own community; assuming our own referrals are representative of most communities, the reader can construct a profile of the case load a genetics center will encounter. Under the second heading (organization) we describe representative programs which can serve individual patient needs and the public health aspects of genetic medicine today (World Health Organization, 1964, 1968). The third section describes those areas of genetic disease particularly susceptible to informed management by the physician and the regional genetics center to which he might refer the patient. In the fourth section we discuss some aspects of diagnosis, counseling, and treatment of patients with genetic disease. The fifth section presents some current information on cost benefits, patients' attitudes, and the yield of our present-day efforts at genetic disease control. The paper is written with the

knowledge that readers of *Progress in Medical Genetics* are to a great extent *not* medical geneticists and represent many branches in the health professions.

SOURCES OF PATIENT REFERRALS

A logical point from which to start is the source of referral for the patient with genetic disease. Where are these patients found and what type of genetic disease do they have? When this is known the deployment of resources can be planned, costs can be calculated and the service for diagnosis, counseling and treatment can be developed in their relevant context. There are three primary sources of patients; the hospital, the consultation or personal referral, and public health (screening) programs. Each is discussed in turn.

The Hospital Admission

Frequency of Genetically Determined Morbidity. Although degenerative disease dominates our view of contemporary medical practice, there is no doubt that gene-determined disease is now an important cause for hospital admission. This is particularly true of admissions to a pediatric hospital. Table 1 shows the results of a survey of a sample from 12,086 admissions to the Montreal Children's Hospital in 1970 (Saginur et al., 1972). About 10% of these admissions are for conditions classified in McKusick's catalogue of Mendelian genetic disease (McKusick, 1971b); another 18% are for congenital malformations (excluding those in the first category) found in the N.I.H. catalogue edited by Hay and Tonascia (1968). About 2% of the total, or a third of the admissions classified as "unknown" (i.e., not clearly belonging in the other three categories: genetic, congenital malformation, and nongenetic), are probably genetic in origin to some degree. Therefore, about one-third of all pediatric patients (medical and surgical) express mutant genes or show results of abnormal gene-environment interaction during intrauterine development. This is a major disease burden, apparently resulting from the 2-5% of live-born infants reported to have congenital or hereditary disease at birth (Stevenson, 1961).

The data for one children's hospital in Montreal are perhaps fairly typical of the problem at large, and Dr. Barton Childs has surveyed the pediatric *medical* admissions to the Johns Hopkins Hospital in Baltimore for 1970 (personal communication). Frequencies in the two studies are comparable even when differences in methods of tabulation and source of sample are taken into consideration. Miller (1964) reported that about 50% of pediatric beds in a British Columbia hospital are occupied by patients with diseases of genetic origin.

The pattern of genetic illness in hospitalized pediatric patients is also of interest. The Montreal study (Table 1) revealed that patients with obvious

TABLE 1.–*Characteristics of Admissions to a Pediatric Hospital in North America**

	Class of admission†	Number of admissions		Total	% of all	Male/ female ratio	Average length of stay (days)
		Medicine	Surgery				
	All			1145	100	1.76	7.31
I.	Genetic AR	18	5	23	2.0	1.3	
	AD	11	12	23	2.0	1.1	
	X	25	6	31	2.7	15.0	
	MF‡	44	1	45	3.9	1.6	
	Chr.	5	0	5	0.4	0.66	
		(103)	(24)	(127)	(11.0)		(7.28)
II.	Congenital malformations§ (excludes group I)	5	205	210	18.4	1.6	8.60
III.	Unknown	34	45	79	6.9	1.1	6.30
IV.	Nongenetic	278	452	730	63.8	1.65	5.70

* Randomized sample of 1145 patients, which is statistically representative of the 12,800 admissions to the Montreal Children's Hospital during the 12-month period May, 1969–May, 1970.

† Abbreviations: AR, autosomal recessive; AD, autosomal dominant; X, X-linked; MF, multifactorial; Chr., chromosomal.

‡ Multifactorial group includes 24 cases of "atopic hypersensitivity" (McKusick, 1968).

§ Group includes 55 cases of hernia, according to classification of Hay and Tonascia (1968).

genetic disease are admitted more frequently to the medical services than to surgical wards, whereas patients with congenital malformations, with rare exception, require surgical admission. There are more males than females among all admissions, but the male predominance is apparent in all "genetic" admissions except for those showing chromosomal defects (where the sample is very small). The average length of hospitalization for *genetic disease* is very close to the average for *all* admissions. On the other hand, 1619 days of hospitalization were required for congenital malformations other than hernia, and only 182 days for the latter. Admissions for genetic disease other than atopic hypersensitivity required 809 days in hospital whereas the latter required 104 days. The combined total is 2714 days; that is, 32.4% of the total number of hospital days were used by patients with genetic diseases or congenital malformations. If this figure is translated into terms of admission costs, 32.4% of 84,384 inpatient hospital days cost 3.31 million dollars (the current bed rate is $120 per day for this hospital). The economic burden for hospitalization of genetic disease and congenital malformations is therefore as impressive as the frequency of such admissions.

Pattern of Mortality from Genetically Determined Disease. The gravity of genetic admissions to hospital is mirrored also by the high percentage of these patients who die and come to autopsy (Table 2). Roberts and colleagues (1970) surveyed the genetic component of child mortality in Great Britain, and from their results and the data compiled earlier by Carter (1956) for 20-year intervals at the Great Ormond Street Hospital for Sick Children, it is apparent that gene-determined illness has increased steadily in this century and now comprises a large portion of pediatric deaths in modern society.

Pattern of Genetic Disease Morbidity. The discharge diagnosis was recorded, according to international convention (U.S. Public Health Service,

TABLE 2.–*Percentage of Pediatric Deaths due to Genetic and Nongenetic Illness in Great Britain*

	Carter (1964)*			
	1914	1934	1954	Roberts et al. (1970),† 1960-1966
Genetic	2.0	1.5	12.0	11.0
Congenital malformations	4.0	4.0	19.0	30.5
Unknown	23.0‡	29.5‡	40.0‡	9.2
Malignancy	3.0	13.5	14.5	8.8
Environmental	68.0	51.5	14.5	40.5

* London area.

† North of England.

‡ Includes diseases termed part-genetic and multifactorial.

1969), for the pediatric admissions reviewed by Saginur et al. (1972). The major diagnoses for "genetic disease" and those for the group of admissions classified as congenital malformations are shown in Table 3. In the former group, multifactorial diseases such as diabetes mellitus and familial atopic hypersensitivity account for many admissions, whereas "classical" hereditary metabolic diseases such as phenylketonuria and renal tubular forms of rickets are virtually absent. This is of course because diseases due to single gene defects are individually rare, although together they constitute a formidable group; but the low figures for phenylketonuria and hereditary rickets in this particular study also reflect the activity of a local program, designed to keep such patients out of hospital (see pp. 169 and 191). Many features of the medical history led the investigators to suspect that the illnesses of the patients classified as "unknown" in Table 1 probably had a significant genetic origin. The fact is worth noticing because these patients are frequently the subject of consultation to the genetics department.

Genetics Consultations at the Medical Center

The Department of Medical Genetics at the Montreal Children's Hospital gets most of its referrals from the wards of the hospital. The requests are usually for aid in diagnosis, by either clinical recognition of a rare syndrome, dermatoglyphic analysis, or study of the chromosomes; because of the clinically oriented

TABLE 3.–*Genetic Diseases and Congenital Malformations Among 1145 Consecutive Admissions to a Pediatric Hospital*

Genetic disease	No.	Congenital malformation	No.
Hemophilia	20	Hernia (all types)	57
Diabetes mellitus	17	Unknown (5)	27
Thalassemia	5	Cryptorchidism	11
Rh disease	5	External ear anomaly	11
Cystic fibrosis	3	Dislocation of hip	6
Christmas disease	2	Hypospadias	5
Phenylketonuria	2	Tetralogy of Fallot	4
Schizophrenia (familial)	2	Craniofacial anomaly	4
Nevus	3	Lower limb anomaly	4
Hypophosphatemic rickets	2	Other (3 each)	12
Craniofacial anomaly	2	Other (2 each)	22
Spastic paralysis	2	Other (1 each)	54
Other (1 each)	27		
Total	92 (8.0%)*	Total	217 (18.9%)*
	Grand total:	309 (26.9%)*	

* Percent of total sample (1145 admissions).

interests of most pediatric residents, the question of recurrence risk is rarely asked. The family is then interviewed, and the question of recurrence risk almost always comes up at this point.

Other families may be referred from the outpatient department, or by physicians outside the hospital and, occasionally, from social service agencies. A few families refer themselves, by either mail or telephone. Since the exact proportions of the various sources of referral will vary with the interests and energy of the physicians in the vicinity, the activity of departmental staff in the wards, and the amount of publicity in the lay press, for example, there is little point in attempting to present precise figures here. A résumé of 349 referrals for counseling accumulated over the past 3 years is shown in Table 4.

Patients with features suggesting a possible inborn error of metabolism are referred for special screening to another unit in the genetics department. This type of referral is discussed more fully in the following section.

"Screening" Referrals from Public Health Programs

About 90% of live-born infants in North America are now screened in government-sponsored programs for one or more of the hereditary metabolic diseases. Many hospitals and regional programs also support special screening programs for the diagnosis of this type of illness in referred patients. There is no need to discuss here the principles and methodology of screening for genetic disease since they have been the subject of many reports in recent years (Scriver, 1965; Hsia, 1966; World Health Organization, 1968; Milunsky et al., 1970; Levy et al., 1971). Screening tests may be simple and of "broad spectrum," such as those commonly used to examine urine metabolites (Buist, 1968; Perry et al., 1966) or blood amino acids (Efron et al., 1964; Scriver et al., 1964), or may be specific and sensitive enough to detect an enzyme deficiency of, for example, hexosaminidase A in the Tay-Sachs trait (Gm_2 gangliosidosis, type I) (O'Brien et al., 1970) or galactose-1-phosphate uridyl transferase in galactosemia (Shih et al., 1971). We can anticipate that such tests will be applied to many people living under a wide variety of conditions and will serve to identify patients who require specific diagnosis, counseling, and treatment. We are assuming that *mass screening* service programs search for diseases susceptible to management.

It is now possible to predict the frequency with which these programs may yield a patient with a particular inborn error of metabolism. Until recently the frequency estimates for many of these diseases were largely theoretical; empirical data are now available from some of the large screening programs (Table 5). These data will allow us to forecast the number of new patients to be found annually in a given community, knowing the ethnic densities in the regions under surveillance and the frequency of the hereditary trait in the particular ethnic group. It is important to recall that the patient yield from

TABLE 4.—*Categories of Diagnosis for 349 Families Referred Specifically for Genetic Counseling over 3 years*

Diagnosis	No.
Autosomal dominant	
Tuberose sclerosis	7
Huntington's chorea	6
Osteogenesis imperfecta	6
Retinoblastoma	4
Aniridia	3
Apert's syndrome	3
Ectodermal dystrophy	3
Ehlers-Danlos syndrome	3
Polycystic kidneys	3
Turner's phenotype (Noonan's)	3
Holoprosencephaly	2
Holt-Oram syndrome	2
Leber's optic atrophy	2
Neurofibromatosis	2
Mandibulofacial dysostosis	2
Nerve deafness	2
Other (1 each)	18
	71
X-linked	
Duchenne's muscular dystrophy	10
Hemophilia	3
Agammaglobulinemia	2
Other (1 each)	4
	19
Autosomal recessive	
Albinism	9
Pancreatic cystic fibrosis	6
Congenital deafness	5
Friedreich's ataxia	4
Werdnig-Hoffmann disease	4
Tay-Sachs disease	3
Thalassemia	3
Cataracts	3
Phenylketonuria	2
Ataxia telangiectasia	2
Chondrodystrophia calcificans	2
Deafness (nerve)	2
Larsen's syndrome	2
Retinitis pigmentosa	2
Riley-Day syndrome	2
Other (1 each)	23
	74
Chromosomal	
Trisomy 21	27
Other (1 each)	5
	32
Nonfamilial syndromes	
Goldenhaar's	3
Leukemia, acute lymphoblastic	2
Rubinstein-Taybi	2
Other (1 each)	7
	14
Miscellaneous	
Consanguinity	17
Racial ancestry	2
Exposure to mutagens or teratogens	2
Other (1 each)	3
	24
Multifactorial or Unclear	
Neural tube defects	20
Mental retardation, nonspecific	15
Multiple congenital anomalies	15
Convulsive disorders	13
Congenital heart defect	6
Limb malformations and mental retardation	5
Microcephaly	5
Delange's syndrome	3
Repeated abortion	3
Cerebral palsy	2
Hemangioma	2
Hydrocephalus	2
Omphalocele	2
Robin's syndrome	2
Other (1 each)	20
	115

TABLE 5.–*Empirical Frequencies of Hereditary Metabolic Diseases (per 100,000 Live Births)*

Disorder	Number tested	Apparent frequency and source of information
Blood screening		
Phenylketonuria	850,501	7.1*†
Atypical forms of PKU	850,501	3.0*†
Benign hyperphenylalaninemia	850,501	3.4*†
Maple syrup urine disease	685,072	0.3*
Homocystinuria	394,635	0.8*
	36,172	2.8†
Hereditary tyrosinemia	251,319	0.4*
	–	30.0‡
Galactosemia	460,766	0.43*
	32,138	6.20§
	141,402	2.8#
Urine screening		
Hartnup's disease	203,959	7.1*
Cystinuria	203,959	5.9*
	4,714	7.8‖
Iminoglycinuria (renal)	203,959	5.9*
Histidinemia	203,959	5.9*
Arginosuccinicaciduria	203,959	0.5*
Hyperglycinemia (ketotic)	203,959	0.5*
Hyperglycinemia (nonketotic)	203,959	0.5*
Hyperlysinemia	203,959	0.5*
Fanconi's syndrome	203,959	0.5*
Cystathioninuria	35,809	5.6**

* Massachusetts, Levy et al. (1971).
† Montreal, Clow et al. (1969).
‡ Quebec, northeastern region, Laberge (1969).
§ Manitoba, Fox et al. (1971).
# New York State, Kelly et al. (1971).
‖ Montreal, heterozygote method, Scriver et al. (1970).
** Australia, Lyon et al. (1971).

public health screening is cumulative. We estimate that the potential case load in North America for patients born with cystinuria, phenylketonuria, histidinemia, and maple syrup urine disease alone is about 675 persons during the first year and 3375 in the fifth year of an ongoing program.

Because government-sponsored participation in the diagnosis, counseling, and treatment of genetic disease is a relatively new development in the field of public health, it seems appropriate to describe in this paper Le Réseau Provincial de Médecine Génétique (The Quebec Provincial Network of Genetic Medicine). This

program emerged under the auspices of state-supported medical care, which has undoubtedly been helpful in the establishment of the Genetics Network in the province. However, it is clearly possible to establish a successful regionalized genetics program outside the umbrella of state medicine. Such programs exist today in many areas of the continent and did exist on a small scale in Quebec even before the present network was established. The Quebec Network is discussed in detail below only because it is most familiar to us.

ORGANIZATION OF MEDICAL GENETICS PROGRAMS

The gap between theory and practice in medical genetics can be narrowed by the availability of appropriate resources for patient care. The objective of a program is to locate patients (diagnosis), advise them of inheritance risks (counseling), and neutralize the expression of the mutant allele(s) (treatment). These objectives will not be achieved effectively if the effort is haphazard. Efficient coordination of resources at all levels is needed if the patient is to gain the maximum benefits of the program. In the following section we will describe the organization of the community-oriented genetics programs with which we are associated. We recognize that they are more likely than not to be representative of other programs in existence but not familiar to us.

Public Health Level

Government-Sponsored Programs. The Quebec Network for Genetic Medicine is an example of a government-sponsored program which was established to control the impact of certain types of genetic disease in the community. The target population comprises all newborns among whom the program searches for several identifiable genetic traits. High-risk groups, in which mutants such as the Tay-Sachs allele(s) and the hereditary tyrosinemia gene(s) are expressed, are also within the purview of the Network.

i. Organization of Network. At a meeting of the pediatric department chairmen from the four medical schools in the province held in 1968, a recommendation to initiate a provincial screening program for phenylketonuria and allied disease was accepted with the provision that the necessary centers for confirmatory diagnosis, counseling, and treatment be established to cover all regions of the province. The program was initiated with government approval in 1969 and became the Provincial Network of Genetic Medicine in 1971. The government of the province provides the funds for the majority of the program's work.

Because of its public health orientation, the Network program was directed initially toward mass screening for certain traits amenable to counseling and treatment. Centralization of resources and coordination of public health services were deemed the best way to provide a service to the relatively few patients

scattered widely throughout the province who would call upon the limited sources of scientific expertise. The initial tactic was to screen the newborn population because of its availability and the need for diagnosis at this age for certain forms of genetic disease. The opportunity to screen for various demographic groups at special risk was not neglected, for example, a region where there is a high prevalence of hereditary tyrosinemia; in the Jewish community in Montreal, Tay-Sachs heterozygote identification is also of interest.

Participation in the program is voluntary. There are no laws which compel a citizen to be tested or to report the presence of genetic disease. Parental consent for testing of the newborn is achieved by an official letter explaining the reason for preventive screening and the need for a blood sample; if no action is taken by the mother, favorable consent is assumed and the sample is taken. Parental objection is honored but is rarely encountered. Approximately 90% of the live births in the province are now screened for certain biochemical abnormalities which may indicate hereditary disease.

The major logistical problem is how to ensure participation of 163 hospitals where infants are born and where the sample for screening are obtained. Each institution is responsible for its own administrative procedures and resource personnel. At the present time 158 hospitals take part regularly in the program, covering about 92% of the live-born deliveries. It took approximately 18 months for participation to reach this level.

A simple filter paper kit was designed for recording pertinent demographic data and collecting samples of whole blood (from a heel prick) and urine. Two centralized laboratories receive and analyze the samples. The specimens are sent to one laboratory where at the present time analyses on blood are performed. The urine-impregnated filter paper, marked with a code number, is sent to the second laboratory.

The central and collaborating laboratories at four university centers are responsible for confirmatory testing and work-up of patients in whom a positive test has been discovered. The large number of specimens handled by the central laboratory requires an efficient and inexpensive communication system for purposes of follow-up. This is done by telephone, or by mail with the aid of colored envelopes easily identified by the postal service in the province. Seasonal conditions which influence delivery of the samples are monitored, and the normal values are adjusted if there is chemical variation influenced by season.

ii. Operation of Network. The funds allocated by the government to the Network amount to approximately $2.50 per newborn. These monies support the cost of: multiphasic mass screening; in-depth diagnosis, counseling, and treatment of patients identified by screening for whom treatment is indicated; research and development (pilot) projects; screening programs in high-risk groups.

The Network is administered by an executive committee consisting of two persons from each department of pediatrics in the four medical schools of the province, two representatives of government, and consultants by invitation. The committee meets at 4- to 6-week intervals to review the operation of the components of the Network program and the progress of all patients under treatment. Innovations in methodology are assessed and priorities for future developments are established. A yearly budget with a report of activities and progress is prepared and presented to the Ministry of Health.

The activities of the Network are carried out through four genetics centers located at the medical schools in the province. Each center utilizes appropriate resources at its own university as back-up for its activities. These regional centers provide complementary technical services to the Network as a whole and complete clinical services for patients in the local geographic area.

The central laboratory, located at one of the regional centers, provides statistical surveillance of the mass screening program through on-line computer facilities, so that constant monitoring of data from the screening program is possible. Seasonal and other sources of change in normal control values are identified, so that the statistical recognition of the abnormal test result (Scriver, 1971b) can be recognized at all times. Lagging participation of particular hospitals and breakdowns in program logistics can also be identified and corrected.

The Network, which has been formally operational for 20 months now, carries out the following projects:

Automated fluorimetric analysis of whole blood for phenylalanine (McCaman and Robins method, 1962), tyrosine (new method), galactose (new method), each in the newborn. Blood sample is collected from a heel puncture onto Schleicher and Schuell 903 filter paper.

One-dimensional partition chromatography of amino acids in the urine of the newborn infant (new thin-layer method). Urine sample is collected on a separate sheet of S and S 903 filter paper.

Uric acid:creatinine ratio in urine of newborn infants (McInnes et al., 1971).

Treatment of patients with hereditary metabolic disease by the methods of Clow et al. (1971); diet is prescribed and medication is provided free of charge to the patient.

Special survey of the Lac St.-Jean region for the true prevalence of hereditary tyrosinemia (Laberge, 1969).

Heterozygote detection program for Tay-Sachs disease control in the Ashkenazic Jewish population of Montreal, using a modification of the method of O'Brien et al. (1970).

Cytogenetics register for statistical and counseling purposes.

Regional tissue culture bank for storage and transfer of fibroblast cultures grown out from biopsies on patients with various genetic traits.

"Research and development" including testing of a new method to screen blood thyroxine levels in the newborn (Laberge, 1972, unpublished data) and a province-wide program for prenatal diagnosis of genetic disease.

Hospital participation in the mass screening project is monitored by comparing the number of test samples submitted to the central laboratory with the provincial statistics for live births. Deficiencies in screening participation are easily identified and can be investigated. Poor participation by a hospital has usually been the result of constraints on available personnel to obtain the samples from the infants before discharge.

iii. Efficiency of Network. Early diagnosis is essential for successful management of many forms of hereditary metabolic disease. The efficiency of sample collection and follow-up for patient retrieval when a positive test is discovered is a critical factor in a genetically oriented public health program. The Quebec Network, which serves 6 million citizens with a birth rate of 90-100,000 infants per year and living in a population belt extending about 900 miles from east to west and about 300 miles from north to south, has special problems to face if centralized testing is to be efficient. The lapsed time for sample collection and analysis was therefore examined (Table 6). The majority (98%) of samples are collected at an age when infants with hereditary disease, such as phenylketonuria, are likely to reveal the abnormal biochemical phenotype. Eighty-five percent of the samples are delivered within 4 days to the central laboratory, which is located close to the population epicenter. The biochemical analysis and data processing were completed in 93% of the samples 5 days after arrival at the central laboratory. This means that almost all patients are less than 3 weeks old when a presumptive diagnosis is made and steps for confirmatory diagnosis and initiation of treatment are taken.

The Network relies heavily on existing public health facilities to retrieve patients in the follow-up of positive tests and to assist with counseling and treatment when required. Communication with patients takes place through the regional health units and, when available, the family physician or medical

TABLE 6.–*Efficiency of Sample Collection and Laboratory Analysis in a Public Health Program**

Days	Age of infant at sample collection (% distribution)	Transit time in mail (%) distribution	Processing time in laboratory (%) distribution
1	0	9.1	0.5
2	1.4	23.2	39.8
3	11.3	28.5	12.8
4	40.3	23.2	23.2
5	37.8	11.3	16.5
6	5.1	4.6	7.0
7	4.0	0.1	0.2

* Average of 11,265 samples received during three 1-month periods at the central laboratory of the provincial genetics Network in Quebec.

specialist. There were many initial difficulties in communication with the public health units, but they are diminishing. Despite these impediments, patient retrieval is efficient, and the average age of patients is 12 days when the diagnosis is confirmed and treatment is initiated.

Public Health Screening by Local Centers. Public health principles can be carried out by local centers, hospitals, and laboratories according to the World Health Organization's recommendations on screening for hereditary metabolic disease (1968). When such screening is practiced, patients will be found. For example, the urine testing program described by Perry et al. (1966) and the plasma screening program of Clow and colleagues (1969) served regional populations. The Montreal Children's Hospital has for many years carried out a battery of chemical screening tests on any hospitalized patient, outpatient, or referred patient at the request of the physician or parent. Approximately 15,000 samples were screened in this manner, about 10% of which revealed some biochemical variation which was either normal (e.g., high β-aminoisobutyric acid excretion in urine [Sutton, 1960]) or indicative of disease. Subsequent follow-up by the referring physician or by the genetics group introduced patients with hereditary metabolic disease into the local center for long-term counseling and treatment. The growth rate of the hereditary metabolic disease clinic at the Montreal Children's Hospital as shown in Fig. 2A and the regional distribution of our patients (Fig. 2B) were determined by our own local conditions. Moreover we believe the data will be representative of the situation at any medical center which provides facilities for diagnosis, counseling, and treatment of genetic disease.

Private Sponsorship of Programs in Medical Genetics

The potential role of medical genetics in modern medical practice has not been ignored by the private agencies. At least two foundations in North America have developed broad programs which are patient-oriented, and many other foundations and agencies are active with respect to specific genetic diseases. The following comments on two general programs known to us are intended to stimulate the reader's interest in this important and rapidly growing interface between genetics and the public at the present time.

*The National Foundation (March of Dimes)** publishes a directory of counseling centers (National Foundation, 1971). A physician or interested party living anywhere in North America may obtain assistance with genetic counseling through this continental directory. The various resources said to be available at the referral centers are indicated in the directory. It should be noted that the criteria for inclusion in the directory have not been defined by the Foundation.

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* The National Foundation (March of Dimes), P.O. Box 2000, White Plains, New York 10602.

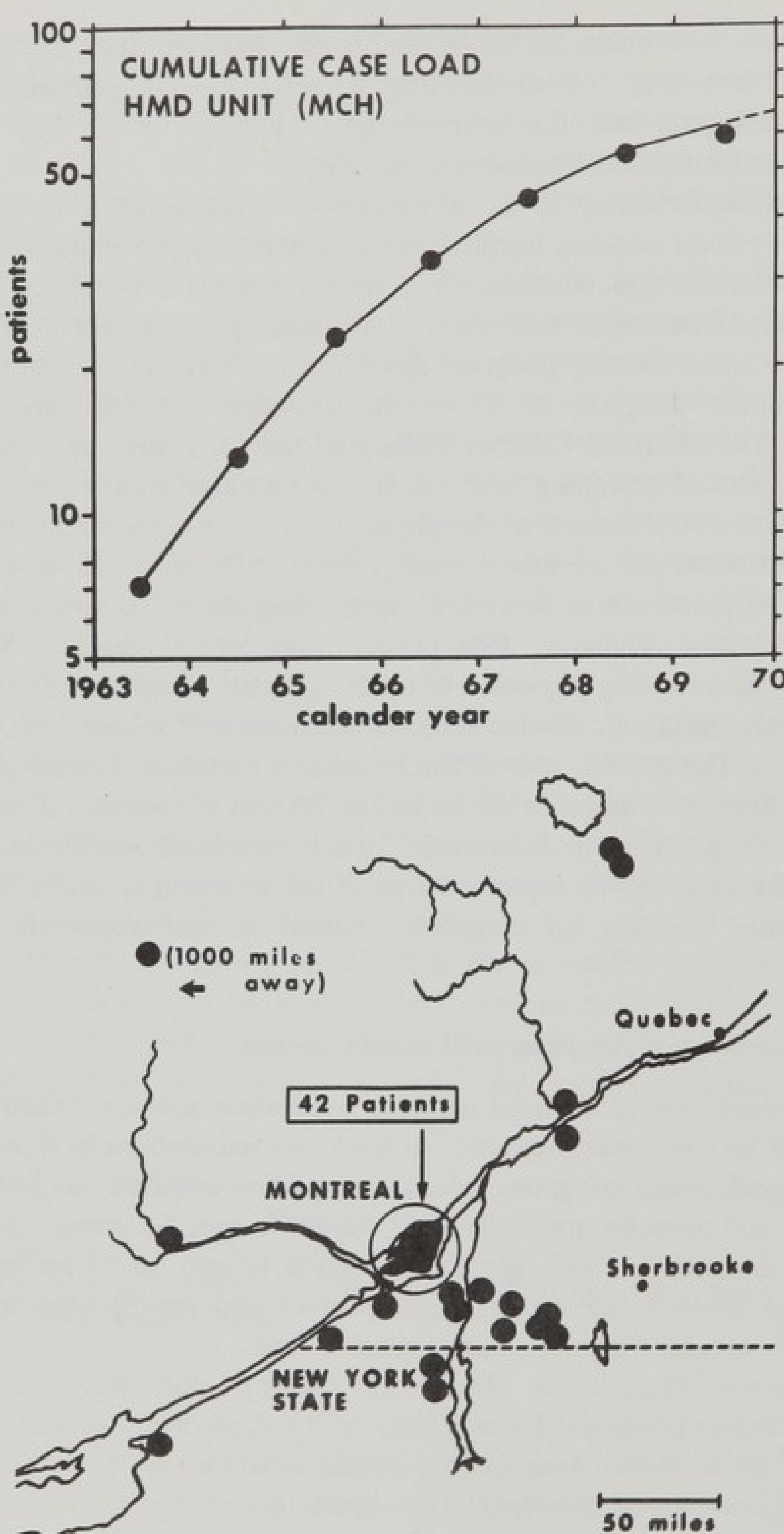

FIG. 2.–A, Growth rate of clinic for patients with hereditary metabolic disease (HMD) at Montreal Children's Hospital (from Clow et al., 1971). B, Distribution of patients in and beyond the Montreal metropolitan area where clinic mentioned in Fig. 2A is located. The wide geographic dispersal of patients with hereditary metabolic disease is characteristic of these patients.

The National Genetics Foundation† sponsors a coordinated network of referral centers for patients with genetic disease. This network also has a continent-wide distribution. There are two types of referral centers in the network:

1. Those that can provide special diagnostic precedures not generally available, as well as technical resources and experience with specific types of genetic disease. These centers, as a group, provide a complementary panoply of tests which cover the current spectrum of hereditary metabolic disease.

2. Those that provide back-up services in counseling and treatment of patients not in need of the specialized investigative procedures provided by the first group. Referral to the appropriate center is performed through the central office of the Foundation. The cost of referral and diagnosis is supported by the Foundation.

Regional Genetics Programs

The regional genetics center with an ongoing commitment to patient care in its community must serve two types of patients with hereditary disease: those with a condition requiring continuous counseling and frequent, complex monitoring of treatment and those who require counseling but no long-term continuous management. The latter could be exemplified by the patient with albinism and the former by the patient with phenylketonuria.

Meeting the Need for Continuous Counseling and Disease Control. The original genetics center, while serving the patient with continuous need for medical care, must endeavor to do so in his normal environment and reduce the time spent in hospital and clinic. At present, there are not enough physicians trained in genetics to meet the demand for patient care in the area of hereditary metabolic disease. Some of the unfortunate results of treatment reported in the recent literature can be traced directly to inadequate supervision of the patient. Alternatives that would avoid such outcomes might include long-term hospitalization of the patients, or more physicians spending more time on these problems. Both these solutions are prodigal of patient time and medical manpower, and another approach is indicated, particularly in view of the "crisis" in the availability of medical manpower (Bergman et al., 1971).

The use of nonphysician personnel to perform most of the health supervision and all of the technical work for management of patients with hereditary metabolic disease has been described by Clow and colleagues (1971). Their approach was predominantly ambulatory, and most of the procedures for monitoring disease control were performed in the home. The organization of their delivery system is depicted in Fig. 3. There are only two full-time

† The National Genetics Foundation, Inc., 250 West Fifty-Seventh Street, New York, New York 10019.

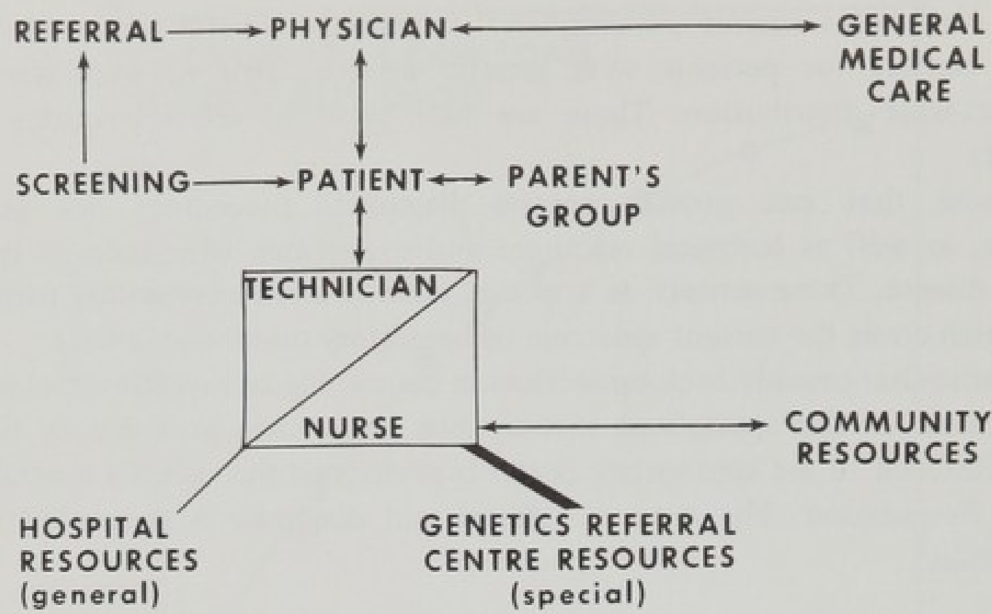

FIG. 3.–A system for supervision of about 70 patients with hereditary metabolic disease requiring diagnosis, continuous counseling, and treatment. The majority (95%) of the specific management is delivered by allied health personnel (from Clow et al., 1971).

supervisors for the daily care of 64 patients with a dozen different types of hereditary metabolic disease; neither is a physician. They utilize a variety of institutional resources for back-up needs and they maintain contact with the patients by telephone, visit, or mail. The family physician, if any, is asked to supervise the health care other than that directly relevant to management of the genetic disease. If there is no family physician, a physician at the medical center assumes responsibility for the over-all health care.

It is estimated that this simple team of allied-health personnel can supervise the management of those patients ascertained by screening a referral population of about 1 million citizens. The demands on the team are naturally determined by the diseases under supervision; different disease profiles would perhaps require teams of different structure and size.

Much of the technical monitoring required for the management of hereditary metabolic disease control may be done in the home; it is usually only the chemical analysis of the sample that needs laboratory facilities. Clow et al. (1971) kept records of how contacts were made for patient supervision and showed that the pattern of patient contact is related to the disease (Fig. 4). Some diseases can be handled by telephone and mail; others require extensive home visiting.

A job analysis of the tactics for delivery of health care in a hereditary metabolic disease unit staffed by two allied-health personnel (Clow et al., 1971) showed that the laboratory-based associate spent 18% of her time talking to patients in person or by telephone and 23% of her time on care-oriented laboratory work. The nurse associate spent 30% of her time talking to and

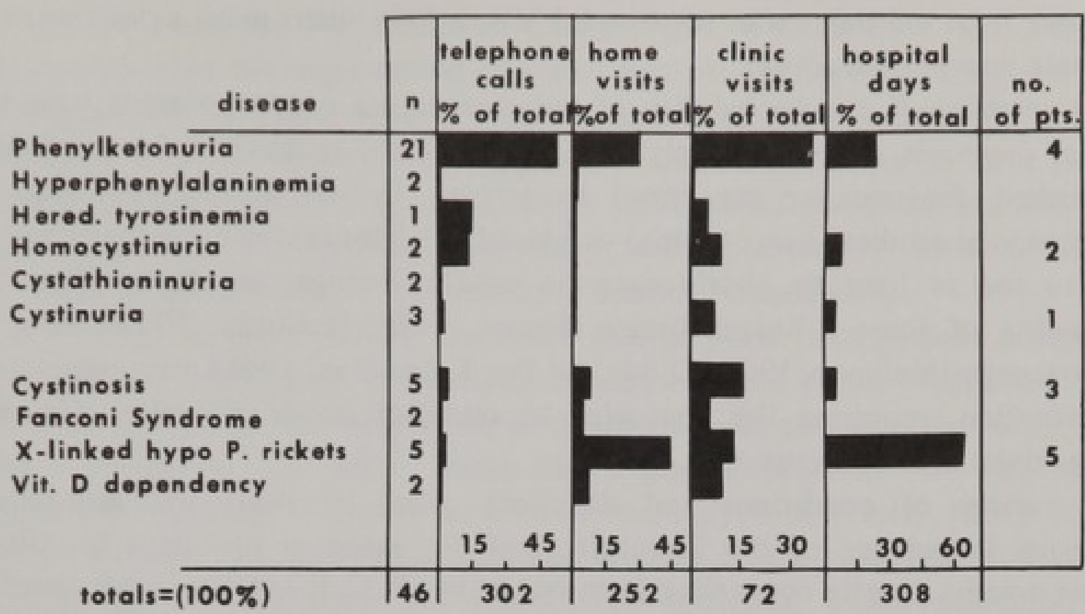

FIG. 4.–Record of patient contacts maintained by team described in Fig. 3. Inborn errors of amino acid metabolism are responsive to management by telephone and in-home monitoring using mail services. Metabolic bone disease requires more home visiting than other types of disease in the group. Hospital days were used almost exclusively for diagnosis of new patients, special investigation, and orthopedic correction of pretreatment deformities in patients with hereditary bone disease. The number of patients involved is given in the last column. (Adapted from Clow et al., 1971).

meeting patients and 21% of the working day traveling to meet the patients in their homes. Both persons spent about 20% of their time recording data and keeping charts on patient progress–an activity which seems to have become almost an anachronism in the realm of much clinical medicine today! It becomes apparent that if these two health personnel did not spend time talking, telephoning, visiting, and charting, the patients and their parents would not be given the continuous advice and support they need. The patient would then need to visit the clinic or hospital, or a doctor would be required instead to visit the home (another anachronism!), or disease control would be less than optimal. None of these alternatives is desirable. Whether the tactics adopted by this particular type of health team can achieve an acceptable result in disease management is discussed below (see p. 191).

Meeting the Need for Intermittent or "Brief-Encounter" Counseling. This type of counseling process has been described elsewhere (Fraser, 1956, 1970, 1971; Motulsky and Hecht, 1964; Carter, 1969b; Thompson, 1967) and will be reviewed only briefly here. It usually begins with someone wanting to know whether a disease will recur in the near relatives of a patient with the disease. Since our consultations usually involve the parents of a sick, malformed, or mentally retarded child, we will assume that this is the case in the following discussion. The role of the counselor is to estimate the risk of recurrence,

interpret it to the parents in meaningful terms, help them reach a decision, and facilitate appropriate action.

To establish *P*, the probability of recurrence, the counselor must have the correct diagnosis. He may simply have to check the reliability of a previously established diagnosis or may need to arrange further investigation such as chromosomal studies (karyotyping) or special biochemical tests. He may even be able to use or have to obtain data on genetic linkage, as can be done for counseling of some X-linked disease (Fraser, 1970; McCurdy, 1971). Genetic heterogeneity (Mellman, 1968; Childs and Der Kaloustian, 1968) may complicate the situation, requiring the counselor to establish which of several clinically similar traits is under scrutiny.

A variety of conditions and situations must be dealt with, requiring extensive knowledge of the literature, and the range of problems for which people sought genetic counseling from one of us (F. C. F.) at our Department of Medical Genetics has already been described in Table 4. (It should be noted that Table 4 does not include 30 families with hereditary metabolic disease counseled by other members of the Genetics Department [C. L. C. and C. R. S.].) We appreciate the fact that the spectrum of diagnoses might be quite different at some other center where there are different skills and interests. Incidentally, 61 of the 370 families, or 16%, have diseases that can be diagnosed by biochemical tests or karyotyping of amniotic cells, or the index trait is sex-linked; in other words, these are conditions where amniocentesis could be relevant.

Taking the family history is a vital part of the process; it may help to establish the diagnosis and in any case is usually essential to the estimation of *P* (Fraser, 1963). A special form is used to ensure systematic recording of the data. It is often necessary to correspond with doctors or hospitals to confirm diagnoses of possibly relevant disease occurring in family members. The first interview usually takes the better part of an hour or more and, in addition to the collection of information, it allows the counselor to begin to get to know the parents, and vice versa. For this reason it is best that the counselor take the family history, rather than an assistant, time-consuming though this may be. It is also preferable to interview both parents since together they may present a more accurate family history than separately, and the counselor has a chance to get some impression of how they interact, which may be helpful later in the counseling process.

The counselor may be able to estimate *P* reliably by the end of the first interview, and if the estimate is reassuringly low, and the parents are happy about it, there may be no need for further interviews. But if the risk is not reassuring, or if the parents have any doubts about it, or show any signs of uneasiness, a second interview is indicated. This may be necessary anyway if records have to be checked, or special tests done. The interval provides a chance for the parents to absorb the information given, clarify their thoughts, and define the questions they want to ask.

Support Facilities for Regional Counseling. Whereas the two foregoing sections describe the organization of actual systems developed to meet the needs of patients with different types of genetic disease, neither system could function efficiently without additional resources available to them. As we have already indicated, no one medical genetics center will have *all* resources, nor will it be able to perform *all* tests (thus the role of a complementary network such as exists under the auspices of the National Genetics Foundation).

A medical genetics center can meet a large portion of patient needs if it comprises a clinical genetics unit whose members have broad experience in the general problems of counseling and in fielding consultations on the patient who "has a syndrome" or who "looks like a genetic problem" or who has an "unknown" disease. Experience with dermatoglyphics (Holt, 1961) can be helpful in some of these situations. The modern center must have cytogenetic facilities and a laboratory for somatic cell genetics, which preferably has experience with more than one type of cell line (e.g., fibroblasts, leukocytes, or organ culture). In the area of biochemical genetics, familiarity with the inherited diseases of amino acid, carbohydrate, and lipid metabolism and neuroendocrine disease is desirable. The inborn errors of membrane transport (Rosenberg, 1969; Scriver, 1969a) have now taken their place, demanding recognition from the consultant. The vitamin-responsive inborn error of metabolism (Rosenberg, 1970; Scriver, 1971a) also requires consideration in this area as in other areas of medical genetics. Obviously a federation of investigators will serve the center's clientele much more effectively than a state of separatism. Moreover, any of these dashing consultants may be forced to eat humble pie in the presence of the patient with a mysterious hereditary metabolic disease, unless he has a friend with a mass spectrometer to find the elusive metabolite which may be the key to the clinical problem. Several authors (Horning and Horning, 1971; Jellum et al., 1971; Mamer et al., 1971) have recently described the diagnostic power of mass spectrometer-gas chromatograph instrumentation for the human biochemical geneticist. In addition the center should probably have an immunogenetics team since many hereditary traits affect host resistance to infectious agents. The list could be extended, being limited only by one's ambitions, available space, and budget.

DISEASE SPECTRUM AND DIAGNOSIS

This discussion began with a consideration of the sources of patients with genetic disease; a description of the facilities which support diagnosis, counseling, and treatment at various levels in the community then followed. At this point a detailed consideration of the disease spectrum that might be encountered by a genetics center and its referral population would be appropriate. However, this is not possible for obvious reasons. The catalogue of

Mendelian traits (McKusick, 1971b) is already large, and it is changing every year (McKusick, 1970, 1971a) (Fig. 1). A catalogue of multifactorial disease would require continuous updating and there would be divergent opinions on its content. There is no catalogue of chromosomal disease, and if it existed, it would also be out of date because new methodology in cytogenetics (Caspersson et al., 1969; Patil et al., 1971) now demands a complete review of the known clinical syndromes. What, then, can the interested person do to obtain reference information on diagnosis of hereditary disease, and what types of diseases merit particular attention in the present state of our knowledge? Admitting that there are several approaches, we have adopted a simple expedient: reference sources which have proven to be most useful in our experience are mentioned; a catalogue of diseases requiring special attention prenatally or perinatally is given; and those diseases susceptible to "environmental engineering" are listed. We anticipate that patients with these conditions will place the greatest demand on the available resources for the application of medical genetics.

It is important first to reiterate three familiar themes relevant to any discussion of diagnosis in medical genetics. These are the problems of expressivity, genetic heterogeneity, and phenocopy.

In every mutant individual, the mutant gene operates in association with a unique selection of other genes and a unique environment. Consequently many of the clinical genetic syndromes vary in their appearance from one patient to the next (expressivity). The clinician must rely on what he sees. A constellation of phenotypic signs will signal the apparent diagnosis to him, and it may not be difficult to distinguish the syndrome when all the features are obvious. But what does the clinician do if the syndrome is only partially expressed? Is the condition in his patient the same as that described in the "classical reference" to which he has turned? He may never know until he can understand what cellular events lie between the gene and the components of the syndrome he is observing. This state of knowledge has been achieved for only a few genes in man; it is no wonder that the clinical geneticist is often hard pressed for a diagnosis.

Genetic heterogeneity (Mellman, 1968; Childs and Der Kaloustian, 1968) introduces further subtlety into the art and science of diagnosis, counseling, and treatment. This is particularly evident now that in vitro techniques are used to study genetic traits (Hsia, 1970). Whereas expressivity refers to the variable manifestations of a single mutant gene, genetic heterogeneity recognizes that different mutant alleles may produce apparently similar phenotypes. Achondroplasia and diastrophic dwarfism, for example, may be mistaken for each other by the inexperienced person. The expert can recognize the clinical differences, and the geneticist knows that these two conditions are different, because the former is dominantly inherited, whereas the latter is recessively inherited. How the different mutant genes produce phenotypes which resemble each other is

unknown. But failure to recognize the important differences would confound counseling in this and many other examples one could choose. Genetic heterogeneity is well known in the hemoglobinopathies, and it should not be surprising that traits such as hyperphenylalaninemia, cystinuria, or galactosemia should be genetically heterogeneous. Consideration and interpretation of genetic heterogeneity is important in medical genetics since it influences the management of disease.

The phenocopy raises yet another problem for the clinician. A phenocopy is an acquired condition which mimics the inherited trait; in the absence of a contributory family history, it may not be apparent whether the patient carries a recessive or new dominant mutation or merely has a phenocopy. There are no phenocopies at the level of the gene product and this complication of differential diagnosis could be resolved if the abnormal cellular mechanisms responsible for the phenotype were known.

General Sources of Information on Diagnosis for Clinician and Counselor

A recent volume of the *Medical Clinics of North America* (Townes, 1969) provides a good overview of genetics in clinical medicine.

Mendelian Traits. The most complete catalogue has been compiled by McKusick. The third edition of the book (1971b) lists 1621 traits for which Mendelian inheritance is apparent or probable. References are supplied for all listings.

Detailed descriptions of the major inborn errors of metabolism are available in the books edited by Stanbury et al. (1972) and by Bondy (1969).

Syndromes. This difficult area is made more accessible through several publications; Smith (1970) has published an excellent book on the subject. Gorlin and Pindberg (1964) describe the syndromes of the head and neck. Gellis and Feingold (1968) have published an atlas of syndromes (mainly hereditary) associated with mental retardation. The National Foundation (March of Dimes)* edits the proceedings of a continuing series of conferences on syndromes; each volume is classified according to a region of the body or system. Cone (1968a, b) has published two interesting compendia of disease, again often hereditary, recognizable by the color of the urine or the odor of the patient.

The wealth of eponyms attached to syndromes has created a deplorable state of confusion, through which Jablonski's *Dictionary of Eponymic Syndromes* (1969) is a handy guide.

Chromosomal Aberrations. As mentioned earlier, there is no modern review of cytogenetics to replace earlier versions published during the preceding decade's exponential phase of growth in knowledge on this topic. New

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* See footnote on p. 173.

techniques for chromosome identification (Caspersson et al., 1969; Patil et al., 1971) will generate many new publications redefining "classical" cytogenetic syndromes. The 1966 "Chicago Conference" classification is still the standard for nomenclature of chromosomal malformation, but the 1971 "Paris Conference" (1972) will soon provide a new one.

Classification of Genetic Disease Particularly Susceptible to Management by Diagnosis, Counseling, and Treatment

Under certain conditions the recognition of genetic disease allows the clinician and geneticist to intervene with an effectiveness which is not possible under conditions of random ascertainment. We consider these conditions to be: (1) prenatal diagnosis; (2) diagnosis at birth; (3) genetic disease responsive to "environmental engineering"; and (4) the high-risk patient in general.

Diseases Susceptible to Prenatal Diagnosis. Milunsky and colleagues (1970) have recently reviewed this subject in a very helpful manner. The diseases listed in Table 7 comprise a catalogue of conditions for which methods are now generally available in most centers well established for diagnosis by amniocentesis and analysis of the amniotic fluid. Information about the abnormal gene product and testing procedures are described in abbreviated form in the review cited.

Diseases Susceptible to Diagnosis at Birth. Many of the inborn errors of metabolism can be identified shortly after birth through manifestations of disturbed metabolic equilibrium, or more directly through enzyme assay. These are the conditions amenable to mass or selective screening. Classification of such disease is constantly changing as new methods are developed and found applicable in the field. A general perspective on all classes of hereditary metabolic disease is available in the new sixth edition of *Duncan's Diseases of Metabolism* (Bondy, 1969). (The trend towards increased emphasis on hereditary metabolic disease, through successive editions of this standard text, illustrates nicely the changing perspectives on the diseases of human metabolism in recent years.) The enhancement of diagnosis and counseling through heterozygote detection is an important component of the management of hereditary metabolic disease. Hsia (1969) provided a helpful catalogue of diseases for which a heterozygous phenotype can be discerned.

Many hereditary conditions cannot be clearly defined with respect to their aberrant cellular mechanisms, but they can be recognized at birth. Bailey (1971) has tabulated such disorders recognizable because of dwarfism at birth. Hecht and Louvrien (1970) listed the diseases in McKusick's catalogue which they considered were detectable at birth. We have used these sources to compile our own catalogue of nonmetabolic conditions (Table 8) which could be identified at birth by the alert clinical geneticist, so that informed counseling could be given as soon as possible in the patient's life.

Hereditary Metabolic Diseases Susceptible to Treatment. This topic has been discussed extensively in recent years (see below). We are concerned here primarily with diseases in which the mutant allele can be neutralized by "environmental engineering" (as distinct from "genetic engineering") (Scriver, 1967a, 1969b). Table 9 lists the diseases for which it is possible to restrict a toxic substance, replace a deficient metabolite, or enhance enzyme activity. The efficacy of such treatment has been carefully evaluated in only a few patients for most of the diseases listed; the catalogue therefore indicates potential treatment more than actual achievement in most cases. O'Brien and Goodman (1970) suggested a useful classification of hereditary metabolic disease likely to produce acute illness requiring special management of the critically ill child.

Diseases potentially responsive to enzyme (gene product) replacement are of great interest since this is the most direct approach to neutralization of the mutant allele. Three modes of replacement have stimulated considerable work which may become pertinent to future patients; none of these forms of treatment could be achieved at present outside a genetics center.

Chang's pioneering work with encapsulated enzymes (1966) still awaits application to human mutants deficient in a specific enzyme. Chang and Poznansky (1968) described replacement therapy in the acatalasemic mouse. However, until some method to bring substrate and enzyme together safely can be devised in man, little more can be done. Stabilization of the catalyst by physical methods (Mosbach, 1970) and a reliable supply of the required enzyme are likely prerequisites if this approach to therapy is to be of practical value.

Others workers have attempted treatment with unprotected enzymes which were thus susceptible to host attack. Hug and colleagues attempted enzyme infusion therapy in metachromatic leukodystrophy (Green et al., 1969) and in glycogen storage disease type II (Hug and Schubert, 1967). In the first instance the enzyme did not reach the brain, where it was needed; in the second the patient developed severe immunological complications which offset the phenotypic benefits. Other groups have infused "naked" enzyme or used a cellular source of gene product and shown a "therapeutic" response. For example, Di Ferrante and associates (1971) increased urinary glycosaminoglycan excretion in Hunter and Hurler patients after plasma infusion, and Knudsen et al. (1971) observed a striking temporary biochemical *and* clinical response when normal leukocytes were infused in place of plasma into one patient with Hunter's syndrome. The latter investigators estimated that infusions a few times annually might have a remarkably beneficial effect on patients with mucopolysaccharidoses.

The potential role of organ transplanatation in enzyme or gene product replacement should not be forgotten. Several attempts have been made to treat immune deficiency diseases and Gaucher's and Wilson's diseases by this approach, with variable success. The major drawback is homograft rejection.

TABLE 7.—*Hereditary Diseases Eligible for Prenatal Diagnosis*

A. At present*	B. Potentially possible in the future†
Disorders of Amino Acid Metabolism	
Argininosuccinicaciduria	Citrullinemia
Cystinosis‡	Hyperlysinemia‡
Homocystinuria‡	
Hypervalinemia	
Maple syrup urine disease‡	
Methylmalonic aciduria	
Ornithine-α-ketoacid transaminase deficiency	
Disorders of Carbohydrate Metabolism	
Glycogen storage disease (type II)	Fucosidosis
Glycogen storage disease (type III)	Lacticacidosis‡
Glycogen storage disease (type IV)	(Pyruvate decarboxylase deficiency)
Galactosemia‡	
Mannosidosis	
G-6-PD deficiency‡	
Disorders of Lipid Metabolism	
Gaucher's disease	Maroteaux-Lamy syndrome
Generalized gangliosidosis	Morquio's syndrome
β-Galactosidase deficiency‡	SanFilippo's syndrome
Tay-Sachs disease (Hexosaminidase-A deficiency)	Scheie's syndrome
Sandhoff's disease (Hexosaminidase-[A+B] deficiency)	
Metachromatic leukodystrophy	
Neimann-Pick disease	
Refsum's disease	
Fabry's disease	
Mucopolysaccharidosis	
Hunter's syndrome	
Hurler's syndrome	
Miscellaneous Inherited Traits	
Adrenogenital syndrome†	Acatalasemia
Lesch-Nyhan syndrome	Chediak-Higashi syndrome
Lysosomal acid phosphatase deficiency	Congenital erythropoietic porphyria
Xeroderma pigmentosum	Cystic fibrosis‡
	I-cell disease
	Oroticaciduria‡
	Sickle cell disease

TABLE 7.–*Hereditary Diseases Eligible for Prenatal Diagnosis (Continued)*

A. At present*	B. Potentially possible in the future†
Chromosomal Aberrations	
Many §	
Congenital Malformations	
	Those recognizable by ultrasound or amnioscopy

Table adapted from Milunsky et al. (1970).

* This list includes only those diseases for which prenatal diagnosis is now clearly feasible; conditions in italics have actually been diagnosed in utero.

† These diseases are considered potentially eligible for prenatal diagnostic procedures, probably in the near future. Diseases are not considered for which the diagnostic criteria are inadequate or for which there is no clinical or medical justification for prenatal diagnosis and its sequelae.

‡ These conditions are known to be genetically heterogeneous and demand special care in diagnosis.

§ About 1 in 200 live births has a chromosomal aberration; the frequency of affected offspring increases with higher maternal age.

Genetic repair of mutant cells after removal from the host, by fusion with a source of the normal gene (Schwartz et al., 1971; Merril et al., 1971) and reinjection into the host, might be put to practical use in the future, as an approach complementary to enzyme replacement.

Such reports suggest that the prognosis for patients with presently untreatable genetic disease may be changing even as this paper is being published. Yet another role for the genetics center will be to keep abreast of developments and apply them when possible to the patients on their roster.

Inherited Traits Placing the Subject at Higher-than-Normal Risk. The term high risk is pleomorphic in its definition, because it means many things to many geneticists. If we put aside its designation for "the near relatives of an affected proband" and for "populations in which a potentially harmful allele occurs at more than usual frequency," "at high risk" could describe the members of our species who have mutant alleles and yet are healthy until placed in an environment to which they cannot then adapt. In such individuals the mutant allele remains silent until some adaptive stress is encountered; the mutation then compromises adaptation and (acute) illness may appear. Previous knowledge of the subject's genotype may be sufficient to avert exposure to a potentially harmful environment. The Afrikaaner with the porphyria variegata allele, the Negro with one hemoglobin S allele, the Arab with G-6-PD deficiency, the

TABLE 8.–*Mendelian Traits in the Newborn Potentially Detectable by the Clinician*

1. Autosomal Dominant Disease

Achondroplasia
Acrocephalosyndactyly (Apert's disease)
Angioneurotic edema, hereditary
Aniridia
Anonychia–ectrodactylia
Auriculo-osteodysplasia
Cataract (some genetic forms)
Cleft lip and/or palate with lip pits
Cleft lip and/or palate with popliteal web
Cleidocraniodysostosis
Cranio-carpal-tarsal dystrophy
Crouzon's craniofacial dysostosis
Deafness with ear pits
Dyschondrosteosis
Ectodermal dysplasia, Clouston type
Ehlers-Danlos syndrome
Elliptocytosis
Epidermolysis bullosa with onychodystrophy
Holt-Oram heart-hand syndrome
Horner's syndrome
Hyperbilirubinemia due to erythroblastosis
Joint laxity, familial
Kok's disease: hypertonia, exaggerated startle reflex
Leri's pleonosteosis
Lymphedema, hereditary (Milroy's disease)
Mandibulofacial dysostosis (Treacher Collins' disease)
Marfan's syndrome
Moebius's syndrome: congenital facial paralysis
Monilethrix
Myotonia congenita (Thomsen's disease)
Nail-patella syndrome
Nemaline myopathy
Oculodentodigital dysplasia
Ophthalmomandibulomalic dysplasia
Ophthalmoplegia, familial static
Optic atrophy, congenital
Osteogenesis imperfecta
Osteopetrosis (Albers-Schonberg disease)
Osteopoikilosis
Pachyonychia congenita
Paramyotonia congenita of Eulenberg
Polydactylia
Ptosis, hereditary
Papillary membrane, persistent
Radioulnar synostosis
Retinal aplasia
Rieger's syndrome, dysgenesis of iris and cornea, hypodontia (and myotonic dystrophy)

TABLE 8.–*Mendelian Traits in the Newborn Potentially Detectable by the Clinician* (*Continued*)

Split lower lip
Split-hand deformity, lobster-claw deformity
Spondyloepiphysial dysplasia, congenital
Supravalvular aortic stenosis
Symphalangism
Von Hippel-Lindau syndrome
Waardenburg's syndrome, white forelock, heterochromia iridis, and deafness
White sponge nevus of the mouth

2. Autosomal Recessive Disease

Achondrogenesis dwarfism
Abdominal muscles, absence of, with urogenital malformation
Adrenal hyperplasia (clinical signs)
Albinism
Alpers' diffuse degeneration of cerebral gray matter
Amaurosis congenita of Leber, I
Amyotonia congenita: see Werdnig-Hoffmann
Anophthalmos, true or primary (some)
Aplasia cutis congenita
Arterial calcification, generalized, of infancy
Asphyxiating thoracic dystrophy of the newborn (Jeune)
Ataxia–telangiectasia
Atrichia with papular lesions
Behr's infantile hereditary optic atrophy
Bloom's syndrome: dwarfism with skin changes
Cartilage-hair hypoplasia
Cataract, congenital or juvenile
Chondrodystrophia calcificans congenita of Conradi
Cockayne's syndrome: dwarfism, marble epiphyses, etc.
Corneal dystrophy, band-shaped
Cretinism, goitrous
Cystic fibrosis of the pancreas (clinical signs)
Dandy-Walker syndrome
Deafmutism and functional heart disease
Deafmutism (some types)
Diastrophic dwarfism
Ellis-Van Creveld syndrome
Epidermolysis bullosa lethalis
Fanconi's pancytopenia, skeletal defects
Glaucoma, congenital
Heart block, congenital
Hemivertebrae, multiple
Hemochromatosis, idiopathic neonatal giant cell hepatitis
Hypotrichosis
Ichthyosiform erythroderma
Ichthyosis congenita
Larsen's syndrome

TABLE 8.–*Mendelian Traits in the Newborn Potentially Detectable by the Clinician (Continued)*

Laurence-Moon-Biedl-Bardet syndrome
Leprechaunism
Macular degeneration of the retina
Marinesco-Sjögren syndrome
Mesomelic dwarfism of the hypoplastic ulna, fibula, and mandible type
Metatropic dwarfism
Microcephaly, some
Muscular atrophy, infantile
Nephrosis, congenital
Osteogenesis imperfecta lethalis
Osteopetrosis
Polycystic kidney, infantile, type I
Pycnodysostosis
Pyridoxine dependency (with seizures)
Reticulosis, familial histiocytic
Rothmund-Thomson syndrome
Sjögren-Larsson syndrome
Spastic diplegia, infantile type
Spastic paraplegia hereditary
Spongy degeneration of central nervous system
Thanatophoric dwarfism
Usher's syndrome
Weill-Marchesani syndrome
Wolman's disease
Xeroderma pigmentosum

3. X-Linked Disease

Albinism, ocular
Albinism-deafness syndrome
Aldrich's syndrome of thrombocytopenia, infection, and eczema
Cataract, congenital, total
Cataract, congenital, with microcornea
Deafness, congenital, perceptive type
Ectodermal dysplasia, anhidrotic
Hydrocephalus due to aqueductal stenosis (some)
Ichthyosis vulgaris
Iris, hypoplasia of, with glaucoma
Megalocornea
Microphthalmia or anophthalmia with digital anomalies
Norrie's disease: pseudoglioma
Ophthalmoplegia, external, and myopia
Retinoschisis
Van den Bosch's syndrome: choroideremia, anhydrosis, skeletal deformity, and mental retardation

Adapted from Hecht and Lovrien (1970), Bailey (1971), and other sources. List excludes the inborn errors of metabolism and emphasizes visible traits.

TABLE 9.–*Hereditary (Metabolic) Diseases Susceptible to Treatment (Dietary or Pharmacological)**

Disease	Mode of Treatment†
Disorders of Amino Acid Metabolism‡	
Argininemia	S, prot
Argininosuccinicaciduria	S, prot
β-Alaninemia	S, prot; V?
β-Methylcrotonylglycinuria	S, prot; V
Carnosinemia	S, prot
Citrullinemia	S, prot
(Cystathioninuria)	(S, prot); (V)
(Cystinuria)	D-Penicillamine
(Cystinosis)	S, prot, cys; dithiothreitol
(Hartnup disease)	(P, prot; V)
(Histidinemia)	S, his
Homocystinuria	S, met; P, cyc; V
Hyperalaninemia	S, prot, CHO; (V)
Hyperammonemia	S, prot
(Hyperdibasicaminoaciduria)	S, prot; P, arg.
Hyperglycinemia (ketotic)	S, prot; V?
Hyperglycinemia (nonketotic)	S, prot; + benzoate
Hyperlysinemia	S, prot
Hypermethioninemia (transient)	S, prot
Hyperprolinemia	(S, prot)
Hypervalinemia	S, val; V?
Isovalericacidemia	S, prot
Maple syrup urine disease	
"Classical"	S, leu; ileu, val
"Intermittent" and "intermediate"	S, prot
"Mild"	S, prot; V
Methionine malabsorption	S, prot.
(Methioninemia) transient	S, prot
Methylmalonicaciduria	S, prot; V
Ornithinemia	S, prot
Phenylketonuria	S, phe
Propionicacidemia	S, prot; V
Hypertyrosinemia	
Neonatal	S, prot; V
Hereditary	S, phe, tyr
Transaminase deficiency	S, phe, tyr
Tryptophanuria	S, try; V
Tryptophan malabsorption	S, prot
Disorders of Carbohydrate Metabolism	
Disaccharidase deficiency	S, lact, etc.
Galactosemia	S, gal
Fructose intolerance	S, fructose

TABLE 9.–*Hereditary (Metabolic) Diseases Susceptible to Treatment (Dietary or Pharmacological)* (Continued)*

Disease	Mode of Treatment†
Glucose, galactose malabsorption	S, glu, gal
Glycogenosis	Various
Pyruvicacidemia (see hyperalaninemia)	
Disorders of Lipid Metabolism	
Hyperlipoproteinemias (various types)	S, lipids; P, triglycerides (various combinations)
Refsum's disease	S, phytanic acid
Miscellaneous	
Oxalosis (glycolicaciduria)	Enz (artifical block)
Hyperuricemia(s)	Enz (artificial block)
Oroticaciduria	P, uridine
Wilson's disease	D-Penicillamine, potassium sulfide
Hypophosphatemic states	P, phosphate
Hereditary hormone deficiencies (e.g., thyroid deficiency, adrenogenital syndrome, diabetes insipidus)	P, hormone
Vitamin-Responsive Traits	
Megaloblastic anemia	V, B_1
Maple syrup urine disease (one variant form)	V, B_1
Lactic acidosis (one type)	V, B_1
Pyridoxine dependency (convulsions)	V, B_6
Cystathioninuria (one form)	V, B_6
Familial hypochromic anemia (one form)	V, B_6
Xanthurenic aciduria (one form)	V, B_6
Methylmalonicaciduria (one form)	V, B_{12}
Hypomethioninemia with methylmalonicaciduria	V, B_{12}
Vitamin B_{12} malabsorption	V, B_{12}
Tryptophanuria	Nicotinic acid
Propionic acidemia (one form)	Biotin
β-Methylcrotonylglycinuria (one form)	Biotin
Pseudodeficiency rickets (vitamin D dependency)	V, D_2 or D_3

* Material adapted from Scriver (1969), O'Brien and Goodman (1970), and Scriver (1971a).

† Abbreviations: S, substrate restriction; P, product replacement; Enz, enzyme replacement; V, vitamin supplementation; term following indicates component adjusted (e.g., prot, protein; his, histidine, etc.) Parentheses indicate possibly benign trait and treatment not required in some patients.

‡ The possibility of genetic heterogeneity for any trait must be considered; this may influence treatment.

Caucasian with α_1-antitrypsin deficiency may all seem healthy until they are exposed, respectively, to barbiturates, altitude, antimalarial drugs, and cigarette smoking. We have compiled a list (Table 10) of some inherited traits whose recognition allows the geneticist and physician to practice preventive medical genetics.

DELIVERY OF CARE TO PATIENTS WITH GENETIC DISEASE

The pattern of genetic disease, the sources of patients, and the structure of genetics programs involved in diagnosis, counseling, and treatment were discussed in the preceding sections of this paper. The methods for delivery of care now merit some attention. Again, we will adopt a framework for discussion based on the level in the community at which the delivery system operates.

Public Health Programs

A program familiar to us, namely, the Quebec Network, illustrates features relevant to most government-sponsored genetics programs.

The initial problem in delivery of genetic services concerns the report of the screening test result. It is not economical or feasible to return an individualized report to all patients, when 100,000 or more tests are performed annually, of which about 98% are normal. To return the reports in batches to the hospitals from which the test samples originated is not the answer, there being insufficient staff to file the results in the charts. Therefore, it is more expedient to report only the presumptive positive tests and to file the data on all tests at the central laboratory. This central archive can be consulted should an individual report be needed.

The mass-diagnosis component of a large public health program must be able to respond quickly to the "signal" (Scriver, 1972) generated by either the sample artifact or the presumptive positive test. Sample artifacts have great nuisance value since they decrease the efficiency of the program and increase its costs. Thus, for example, insufficient sample loading or spoilage and contamination of the filter paper vehicle have been a significant artifact at the beginning of all mass screening programs employing the filter paper method for blood spot screening (Guthrie and Susi, 1963; Levy et al., 1968a; Burns and Vanderlinde, 1971) or urine screening (Levy et al., 1968b; Levy et al., 1969). In the beginning the screening program in Quebec found 18.3% of its samples to be inadequate and participation in the field was only 30%. After 18 months of operation, the same program recorded 92% participation and only 2 or 3% of the analyses were unsatisfactory. This improvement was achieved not only through a persistent demand for better participation but also through the instruction of participants in the field on how to collect the samples. It is important to obtain a reliable first sample because follow-up testing is inefficient. A pilot program (36,000

TABLE 10.–*Some Inherited Disorders Placing the Patient at Altered Risk* in Particular Environments*

Trait or system affected by the mutation	Environment or condition	Effect in patient at risk
Liver Enzyme		
O-Dealkylation	Acetophenetidin	Hemolysis and methemoglobinemia
Microsomal "oxidases"	Dicumarol (antipyrine, phenylbutazone)	Hemorrhage
"Specific" hydroxylase	Diphenylhydantoin	Dilantin toxicity
INH transacetylase	Isoniazid (sulfamethazine, hydralazine, phenelzine)	Polyneuritis
Glucuronide transferase (Gilbert's disease; and Crigler-Najjar syndrome)	Salicylates, tetrahydrocortisone, menthol	Jaundice and drug toxicity
Porphyria variegata and hepatic type	Barbiturates	Increased porphyrin synthesis; acute "attack"
Atypical alcohol dehydrogenase	Alcohol	Increased alcohol tolerance
Plasma Enzyme		
Pseudocholinesterase	Succinylcholine	Apnea
Erythrocyte Enzyme		
Diaphorase (methemoglobin reductase)	Nitrites, sulfonamides, acetanilide, amines	Methemoglobinemia
Glucose-6-phosphate dehydrogenase (some mutations)	Primaquine, sulfonamides, etc.	Hemolysis
6-Phosphogluconic dehydrogenase		
Glutathione reductase		
Hemoglobin		
Hemoglobin S (heterozygote)	Hypoxia (e.g., with anesthesia, altitude)	Intravascular sickling
Hemoglobin H	Sulfonamides, nitrates	Hemolysis
Hemoglobin Z	Sulfonamides, primaquine	Hemolysis
Methemoglobins	Sulfonamides	Hemolysis and methemoglobinemia
Miscellaneous Traits		
Hereditary resistance to coumarin anticoagulants	Warfarin	↓ Response to warfarin ↑ Response to vitamin K

TABLE 10.–*Some Inherited Disorders Placing the Patient at Altered Risk* in Particular Environments* (*Continued*)

Trait or system affected by the mutation	Environment or condition	Effect in patient at risk
Acatalasia	Hydrogen peroxide	Mouth ulcers
Pentosuria	Aminopyrine, menthol	↑ Xylulose excretion
Phenylketonuria	Catecholamines	↑ Pressor response
Familial dysautonomia	Catecholamines	↑ Pressor response
Down's syndrome	Atropine	Sensitivity to drugs
Glaucoma (narrow-angle type)	Mydriatic drugs	Acute attack
Glaucoma (trabecular type)	Corticosteroids	Acute attack
Muscular subaortic stenosis	Digitalis, etc.	Output failure
α_1-antitrypsin	Smoking	Pulmonary emphysema
Postanesthesia hyperthermia	General anesthetics	Hyperthermia and hyper-rigidity
Abnormal response to CS_2 exposure	Carbon disulfide	Polyneuritis

* The trait usually increases risk for illness; occasionally an improved risk is observed (e.g., with variant liver alcohol dehydrogenase). Substances in parentheses should produce effect in trait, but actual records of exposure are not known. Table adapted and expanded from LaDu (1969).

families) with extensive publicity and "indoctrination" of the mother could achieve no better than 62% follow-up for repeat testing on a scheduled basis (Clow et al., 1969). The Quebec program fails to obtain a repeat test in about 50% of cases, where follow-up is indicated mainly for technical reasons. Follow-up of presumptive positive tests is more successful, probably because greater efforts are made when a patient with apparently potential disease is at risk.

The mean and standard deviations for 57,517 phenylalanine determinations in the first 9 months of 1971 were 2.63 ± 0.46 mg%, respectively. Therefore +3 SD above the mean value for phenylalanine is 4.01 mg%. There were 49 first tests for blood phenylalanine content yielding results above 4 mg %. We have reviewed the retrieval rate on these presumptive positive tests. The number of second samples obtained is expressed as a fraction of the "positive" tests in the range of phenylalanine concentration given: 4.0-4.5 mg %, 7/23; 4.5-5.0 mg %, 7/16; 5.0-5.5 mg %, 3/4; 5.5-6.0 mg %, 1/1; 6.0 mg %, 4/5. This means that 22 of 49 tests were followed up; 1 case each of phenylketonuria and benign hyperphenylalaninemia was found in this portion of the survey.

The interpretation of the "positive" test (Hsia, 1966; Scriver, 1967b, 1972) is an important activity of any public health screening program. For example, when mass screening for phenylketonuria began in earnest about a decade ago, it was believed by most people that only phenylketonuric patients had hyperphenylalaninemia; thus when hyperphenylalaninemia was found it would indicate a new case of phenylketonuria. This logic was soon shown to be false because extensive genetic and phenotypic heterogeneity accounted for the hyperphenylalaninemic trait in man (Rosenblatt and Scriver, 1968; Hsia, 1970). It is safe to say that the positive test always requires careful interpretation before a diagnosis is assigned and counseling and treatment (if necessary) are undertaken. No one is happy if the screening net misses a patient who subsequently develops the disease which was the target of the program, but, by the same token, no one can be very pleased if a patient *without* the index disease is wrongly diagnosed and treated unnecessarily, or even harmfully. It is for this reason that the public health program should encourage referral of the patient with a positive test to a center familiar with the problem and prepared to undertake further investigation. The referral can be made through the physician, by the local health unit, or by the central laboratory—whichever is best for the patient.

The direct responsibility of the public health program to the patient may end when the regional center takes on the management of the problem. In the Quebec program, this is not the case because a central register of patients is maintained wherein progress reports are deposited annually; moreover, the genetics Network supports the total costs of treatment at the regional centers, and operates the centers.

Regional Programs

Two different forms of patient care are called for at the regional center to which patients with genetic disease are referred. The first is the traditional type of genetic counseling; the second is treatment of disease coupled to "continuous" counseling. The former requires skills and experience quite different from the latter and each serves rather different objectives; the demand for both types of service will increase in the future as a direct result of the heightened interest in genetic disease.

Counseling of Problems Which Do Not Require Long-Term Management. The diseases which call for this type of counseling are on the whole more common than the inborn errors of metabolism (Carter, 1969a) and, moreover, the phenotypic relationships with the mutant gene are often less clearly understood. In general, disease which can be handled by one or two interviews and counseling sessions either has a low recurrence risk or does not require long-term management.

The counselor has several objectives to meet if the patient is to receive sufficient information in order to make informed decisions. The task begins by communicating an awareness of the *probability of recurrence* (*P*) of the hereditary illness.

i. Establishing P. The process by which the counselor establishes the recurrence risk in question has been reviewed elsewhere (Fraser, 1970; Murphy and Mutalik, 1969). It involves placing the disease in one of four etiological categories: diseases due to major mutant genes, chromosomal aberrations, major environmental agents, and multifactorial causes. The recurrence risk can then be calculated either from the Mendelian laws or by selection of the appropriate empirical estimate.

ii. Interpreting P. The next step is to make sure the parents know what the probability figure means in their own situation. Some have trouble with the very concept of probability, and some, even though they have an affected child, may not grasp the full implications of having another one.

iii. Decision. The counselees have probably not asked for an estimate of *P* just out of curiosity, but will want to make some sort of decision–whether to have another baby, seek sterilization, marry, adopt, or whatever their particular problem involves. The counselor may help the parents make a wise decision. He can point out the various factors to consider, such as the severity of the disease, its impact on the rest of the family, the social, economic, and religious pressures, and the possible solutions.

iv. Taking action. The decision reached may demand definitive action, such as referral for contraceptive advice, sterilization, artificial insemination, adoption, or sometimes abortion.

Prenatal diagnosis by amniocentesis now provides a solution in the case of a small (but increasing) group of diseases, allowing parents the privilege of having unaffected children, if they are willing to take the option of having the affected ones aborted.

v. The follow-up. A follow-up interview is desirable for several reasons. It may reinforce the parents' understanding of the information given and correct any misapprehensions resulting from reinterpretation of the information. Not infrequently follow-up interviews reveal that the figures given have been modified upward or downward, either through reinterpretation with the "aid" of friends and relations, or perhaps in response to their own wishes, subconscious or otherwise. For instance, when a one-in-four recurrence is translated into a three-to-one risk, one wonders if this reflects the parents' desire not to have further children, for any reason.

Second, the follow-up may reveal attitudes about the problem and the counsel given that will help the counselor to improve his practice.

Our follow-up studies have suggested the following principles.

1. The counseling should not be done too soon after the diagnosis is made, else the parents may be too upset to hear what the counselor is saying.

2. The approach must be adapted to the parents' level of education and more particularly to their capability of understanding the information they are given.

3. It is preferable to interview both parents. This tactic will provide more reliable data and it forestalls the possibility that the information will be "slanted" as it is passed from one parent to the other. On the other hand, it is helpful to allow some time to talk with each parent separately, which may bring out additional information and allows expression of attitudes or information that one parent may not care to reveal to the other.

4. Charts and other visual aids are helpful in getting the message across.

5. The facts given should be written down and placed on record, in one or more of the following: the patients hospital chart, a letter to the family doctor, or a letter to the parents.

6. The counselor should not try to impose his view of the appropriate decision. However, it is not enough to present the required probability and leave it at that. In the ensuing discussion the parents may ask the counselor what he would do in the same situation, and in these circumstances he should say, as best he can, what he thinks he would do. More than one of our counselees has, in retrospect, expressed a wish that we *had* been more directive, at least in emphasizing what the fact of an affected child meant, in terms of daily living. "I understand the statistic in my head, but I don't *feel* it."

Finally, there are situations where a mutant gene or chromosomal rearrangement segregating in the family makes certain relatives high-risk individuals. Such things as Huntington's chorea and multiple polyposis of the colon (dominant), hemophilia and Duchenne's muscular dystrophy (sex-linked recessive), and chromosomal translocations may denote certain family members other than the parents as having a high risk of developing the disease or having affected children. Seeing to it that these individuals receive counseling may be troublesome, involving problems of breach of confidence or invasion of privacy. But the counselor may also be criticized for failing to provide information that could possibly prevent a tragedy, and with the help of the counselees and the appropriate family physicians, the task can usually be done.

Our family follow-ups have reassured us that it is false to assume that "people are going to go ahead and have children no matter what you tell them." It is true that some parents seem willing to take what seems to us a rather inordinate risk, and some ignore the genetic hazards to the point of irresponsibility. But the majority do heed the risks, and our findings agree fairly well with those of Carter et al., (1971) that when the risk of recurrence is low (less than 10%) most parents are prepared to take a chance, but when the risk is high the majority take steps (however imperfect) to stop having children. Our results are more encouraging than some of those reported by others. It is important to distinguish between parents who have been counseled routinely, as part of, say, a clinic procedure, and those who have actively sought counseling. In the former group,

understanding may be poor, and the information may be ignored because it was not requested. In the latter group the majority are grateful for the knowledge, and heed it, making the counselor's task rewarding.

Counseling of Hereditary Disease Requiring Long-Term Management. Active treatment of certain types of genetic disease has become an important facet of modern medical practice. The principles are simple. Neutralization of the mutant allele may be possible with so-called "environmental engineering," employing four basic procedures, alone or in combination: (1) substrate restriction, if substrate accumulation is the principle cause of phenotypic pathology; (2) product replacement, if depletion of a metabolite is important; (3) coenzyme supplementation, if the activity of the mutant enzyme can be enhanced by its coenzyme; and (4) enzyme replacement, if possible. Several reviews of principles and practice in this area of applied genetics have appeared in recent years (Scriver, 1967a; Craig, 1968; Scriver, 1969b; Holtzman, 1970; Dancis, 1970).

The effectiveness of the counseling and treatment for this type of genetic disease is directly proportional to the degree of patient contact. We stress that such disease in general has a low frequency and a high risk of recurrence in the affected family. The tactics for establishing and maintaining good communication with the patient and his family were described earlier (see p. 182). The results of supervision are the ultimate test and an analysis of performance of one program counseling hereditary metabolic disease patients has now been reported (Clow et al., 1971). There were 64 patients with a dozen different types of hereditary metabolic disease under supervision. The effectiveness of management was evaluated from two diseases in particular, both of which are prototypes of hereditary metabolic disease.

In the case of classical phenylketonuria, it was noted that telephone and mail services played a major role in the establishment of in-home disease control. It was possible to maintain 16 patients safely on a low-phenylalanine diet (substrate restriction) for long periods of time (Table 11). Frequent monitoring of the phenylalanine concentration in blood samples taken by the parents or a visiting nurse provided an index of biochemical control. Somatic growth of these patients was satisfactory in all cases indicating avoidance of hazardous overtreatment (Hanley et al., 1970). The most important index of successful treatment of these patients was their mental development. The patients diagnosed at birth and treated from very early infancy did well (Fig. 5) in comparison to those diagnosed and treated later in life. These results indicate that it is possible to apply the principles of substrate restriction as a means of neutralizing a mutant allele to patients on a domiciliary basis. Other groups where careful and consistent patient contact is maintained also report good results in the treatment of phenylketonuria (Sutherland et al., 1970; Grady et al., 1970; Hunt et al., 1971).

TABLE 11.–*Plasma Phenylalanine Control in Phenylketonuric Patients on Low-Phenylalanine Diets**

Number of patients on treatment		16
Number of months treated per patient	Mean	21
	Range	1-30
Number of blood samples taken	Total	491
	Average per patient	31
	Monthly average per patient	1.5
	Range	1-7
Plasma phenylalanine concentration (mg %)		
For all samples (mean ± SD)		10.2 ± 4.6
Range of means per individual patient		6.1 – 15.1

* Treatment effect is monitored by determining the plasma phenylalanine concentration. The blood samples are obtained in the home by the parent or visiting nurse with a kit (Clow et al., 1969). The kit containing samples is sent to the genetics center, where phenylalanine concentration is measured by a partition chromatographic method (Scriver et al., 1964). Phenylalanine content of diet is adjusted according to the result of the test.

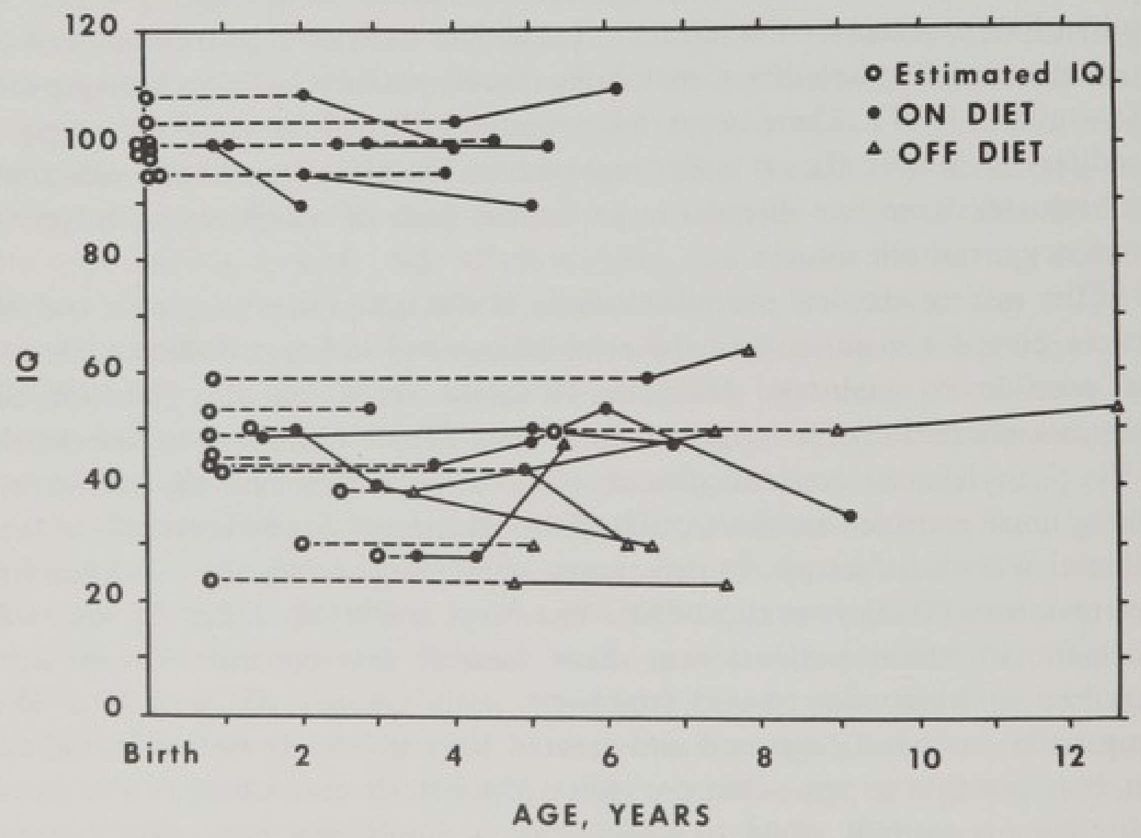

FIG. 5.–The I.Q. in patients with "classical" phenylketonuria. Those treated from birth have normal I.Q. values; a delay in diagnosis and onset of treatment is associated with impaired I.Q. attainment. The earliest value for each patient is estimated in retrospect from the subsequent values which were obtained by formal testing.

X-linked hypophosphatemia is another prototype of the "difficult" hereditary disease. Although long known as "familial vitamin-D-resistant rickets," it has recently been shown (Arnaud et al., 1971; Glorieux and Scriver, 1972) that the apparent vitamin D resistance does not have any relationship to a defect in calcium metabolism; a primary disorder of inorganic phosphate conservation is apparently the major determinant of the phenotype in this disease. If that is the case, phosphate replacement to replenish the depleted phosphate pool is indicated. This treatment is not simple, and the literature records more failures than successes. Nonetheless, with constant in-home support through frequent home visiting, treatment can raise the serum phosphate concentration almost into the normal range and maintain it there for weeks on end (Clow et al., 1971; Glorieux et al., 1972). When this is achieved the rickets heal completely, and of even greater significance, the longitudinal growth velocity for total body length and for the lower limbs is accelerated well above normal average growth rate. This means that dwarfism and bone deformity could be prevented by early diagnosis and treatment of this vexing X-linked trait. We believe the most important determinants for this exceptional therapeutic response are the constant monitoring of phosphate therapy and the support given to the patients in the home; this management can be performed by allied health personnel (Clow et al., 1971).

It should be stressed that the two diseases described here are used only to illustrate the achievement possible in an integrated program which provides aggressive diagnosis, continuous long-term counseling, moral support, and close supervision of the biochemical response to treatment. There is good reason to believe that this approach to management of hereditary metabolic disease could close the gap between theory and practice for many of the potentially treatable diseases mentioned in Table 9 and beginning on p. 181.

Counseling of Patients at Special Risks. Two situations dominate the interest of human geneticists dealing with patients at special risk, namely, the circumstances of prenatal diagnosis, and certain groups, ethnic or otherwise, where harmful mutant alleles appear with greater frequency than usual.

i. Prenatal diagnosis. The subject of prenatal diagnosis for genetic disease has been covered widely and in depth in the literature. There have been several excellent general discussions of the principles which direct our current efforts at prenatal diagnosis (Nadler, 1968, 1969; Littlefield, 1969; Anonymous, 1969; Edwards, 1970; Littlefield, 1970; Emery, 1970; Nadler and Gerbie, 1970; Milunsky et al., 1970; Bergsma and Motulsky, 1971).

The technique of transabdominal amniocentesis by which amniotic fluid is obtained for diagnosis is the key clinical procedure (Nadler, 1969; Edwards, 1970). Skill is required to obtain the sample and timing is important. The sample must be obtained without harm to fetus or mother and the fluid should be free of blood. Amniocentesis is not yet a procedure to be performed lightly or in the

doctor's office. Moreover, the material obtained must be utilized to the utmost. A competent laboratory must be prepared to receive the sample and to perform the analysis desired. Since important decisions are at stake, laboratories with only casual experience in the performance and interpretation of tests for genetic diagnosis should not be involved in the handling of amniotic fluid. Furthermore, the concentrations of metabolites and the activities of enzyme in amniotic fluid and its cells are peculiar to that fluid and those cells, and the laboratory must know the normal range preferably by its own standards, before it is prepared to identify an abnormality (Kaback, 1971). Contamination of the fluid by red blood cells and maternal cells can also complicate diagnosis.

Prenatal diagnosis is a procedure which should be done only at a center equipped to cope with the problem and which keeps in touch with other centers participating in this rapidly growing field. There is a movement in progress, under the auspices of the National Institute of Child Health and Development, to maintain a register of amniocentesis performed in North America. The purpose of this is to monitor the benefits and hazards of the precedure both for disease control and for unaffected offspring exposed to amniocentesis. Whereas most attention is focused on the fetus at risk for diseases in which early diagnosis and management in utero are critical (Milunsky et al., 1970), it is important to realize that the majority of offspring subjected to the procedure will be unaffected by such diseases. Their safety must be assured. The permissive trend in attitudes toward the use of prenatal diagnosis should provide the appropriate climate for critical judgment of benefits and hazards during the period when those techniques are being learned and applied on an ever-increasing scale.

ii. High-risk groups. Besides specifically identified pregnancies where the fetus is at high risk for genetic disease, there are many other examples of the high-risk patient, for whom benefits from applied genetic medicine can be anticipated. A recent editorial (Mentzer et al., 1970) described the clinical importance of knowing who the carriers are for the sickle cell trait. Until recently, many would have thought that screening for sickle cell carriers was inappropriate; some even protested that tones of racism would pervade any such venture! But when a society has a large population in which 8-14% of its members have the sickle cell trait, and when that society inducts these people into its military operations where 1% of the carriers of the trait die from a sickle-cell crisis induced by the military training maneuvers, then that society should be taking steps to identify the individuals at risk and protect them from adverse conditions. If society is willing to pay for the recruit's military training, it surely can afford his hemoglobin electrophoresis, in this instance. Of equal importance to the Hb-S heterozygote are the hazards of air travel, anesthesia, and athletics at high altitude. This exceptional example brings out many of the issues which impinge on decisions concerning the management of high-risk groups.

Not all carriers for hereditary metabolic disease are at this type of risk. Then why bother about identifying carriers? The answer is found in logistics. If the homozygote frequency is 0.0001, the carrier frequency is 0.02. Heterozygote detection is thus an effective way to prevent disease if the carrier can be counseled to prevent diseased offsping from being born (Motulsky et al., 1971; Hsia, 1969). In appropriate circumstance, this is possible with prenatal diagnosis and therapeutic abortion in affected offspring. The prototype of this principle applied to the community is screening for Tay-Sachs disease. The frequency of Tay-Sachs disease is believed to be about 1/3600 in Ashkenazic Jews, that is about 10 times more frequent than in other populations. The carrier prevalence is therefore about 1/30, or about three times more frequent than elsewhere. A method is now available (O'Brien et al., 1970) which can efficiently discriminate heterozygotes with partial deficiency of serum hexosaminidase A (the deficient enzyme in Tay-Sachs disease) from homozygous normal subjects. Consequently, it should be possible to screen all young persons and all parents in the childbearing period of life and to identify those couples at risk for the birth of homozygous Tay-Sachs offspring. The risks can be explained; should the couple elect to have children, the pregnancy can be monitored; if the fetus is affected, a therapeutic abortion could prevent the appearance of a doomed child. O'Brien and colleagues (1971) have recently published their experience with the monitoring of pregnancies at risk for this disease; about 95% of the parents who were told their offspring was affected elected to have an abortion. Community-wide programs to control the appearance of Tay-Sachs disease are in progress in Baltimore, Montreal, and Toronto.

It is not appropriate to discuss all possible examples of high-risk groups which could benefit from diagnosis, counseling, and management. The reader might, however, consider the feasibility and the consequences of any effort that could be made on behalf of high-risk groups known to himself.

CONSUMER RESPONSE TO APPLIED MEDICAL GENETICS

Although producer and consumer are not always in accord on the priorities of the medical industry, the consumer is always interested in the industry's product. What is the status of the product available for the patient with hereditary disease?

Attitudes of the Government-Consumer

"He who pays the piper, calls the tune."

A "medicare" society must do its cost accounting to discern whether screening for genetic disease merits the effort and cost of the program; it may also scrutinize the costs of treatment to find out where the savings, if any, occur.

Some modest experience of our own with the cost benefits of genetic medicine will serve to illustrate this theme.

Cost-Benefit Profiles. A schematic presentation of the benefits which can accrue when the principles of diagnosis, counseling, and treatment are applied to patients with genetic disease is shown in Fig. 6. The relative cost of a hereditary trait should be considered in biological terms (the most significant for the patient) and in terms of the economic burden to the consumer and society.

i. Cost profile for untreatable severe illness. The cost of such disease is very great in human terms (Curve A, Fig. 6). When the condition is Tay-Sachs disease or sickle cell disease, for example, the economic aspects are also significant. It is this type of hereditary disease for which prenatal screening and management have much to offer. The human cost can be lessened and the economic burden can be reduced by monitoring of the pregnancy and providing the option for abortion of the affected fetus (Curve A′). In some cases this negative approach can be replaced by more positive management, as in erythroblastosis.

The actual cost benefits for monitoring the pregnancies of all women over 35 years of age and the increased risk for an offspring with the trisomy 21 (Down's) syndrome have been calculated for a community of defined size by Milunsky et al. (1970). Assuming certain facts about the life expectancy of the patient with this syndrome, the cost of institutional care for the retarded and the likelihood that this mode of care will be used at some time in the patient's life, the cost of medical care for 1 patient with mongolism during a lifetime is

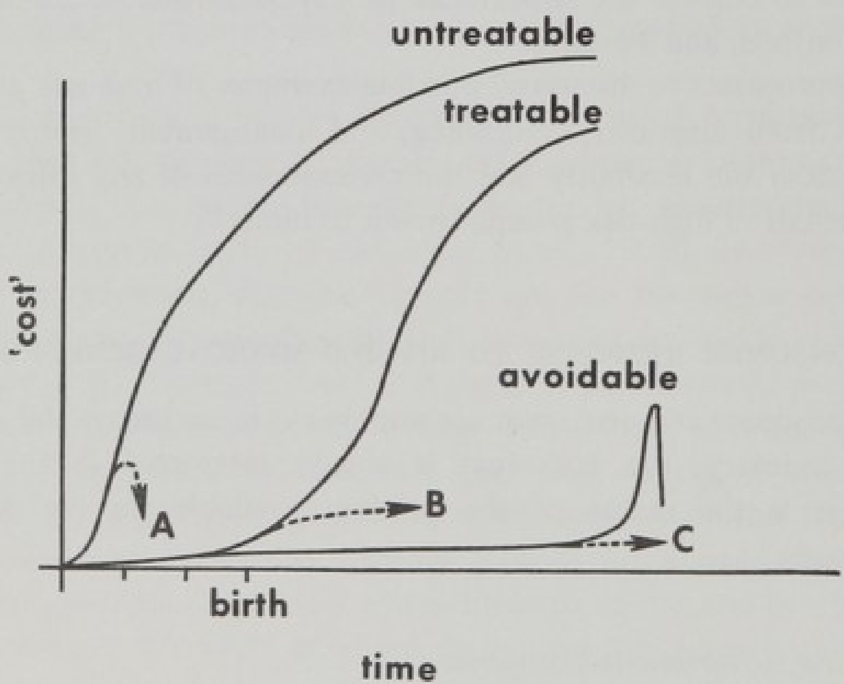

FIG. 6.–Relative cost profiles for untreatable (curve A) and treatable (curve B) genetic disease significantly affecting health. Curve C represents the relative cost of illness resulting from individuals placed at risk in special environments. Early diagnosis, counseling, and treatment can alter the cost profile of each type of inherited disease.

estimated to be on the order of $60,000. About 165 pregnancies would need to be monitored for each case of trisomy 21 found. This means that if society could afford $360 per pregnancy, in women over 35, it could prevent the birth of the children with mongolism, provided all such women at risk were monitored and the option of abortion was used when a fetus with Down's syndrome was identified.

ii. Cost profile for treatable disease. Mutant alleles which are harmful to the patient (Curve B, Fig. 6) but which can be neutralized achieve an improved cost profile with early diagnosis and treatment. The cost for diagnosis and treatment of phenylketonuria in North America is about one-third of the corresponding cost for care, should the patient not receive treatment and require institutional care because of mental retardation. Moreover, the treated patient is likely to become a productive member of society.

iii. Cost profile of "avoidable" hereditary disease. Those patients at risk in certain environments (viz., Table 10) will benefit from knowledge of their phenotype. The cost of a life-threatening or temporarily incapacitating illness (Curve C, Fig. 6) can be avoided by the appropriate diagnosis and counseling.

Government will not be attracted to programs of preventive medicine in which the cost savings attributed to early diagnosis are totally consumed or overexpended in a treatment program. An inadequate treatment program may even yield an ineffective member of society bringing no gain to anyone. It has been estimated that treatment costs can be as low as $2 per patient day in programs providing constant supervision of ambulatory patients with hereditary metabolic disease (Clow et al., 1971). Moreover, it is possible to meet an increasing demand on clinic resources or hospital beds (Fig. 7) if the regional genetics center structures its counseling and treatment program efficiently. The annual saving in hospitalization costs in one government-sponsored regional program (Clow et al., 1971) neutralized half the cost of its own operation; cost savings of this order are likely to catch the eye of any budget officer!

Governments may also become interested in the genetics of populations at risk. For example, the quarter-million inhabitants of the Lac St.-Jean region of Quebec are the progeny of a few hundred settlers who immigrated to the region 2-3 centuries ago. A "founder effect" for at least one mutant allele (Laberge, 1969) has been identified in the region. The hereditary illness involved is usually fatal and very demanding on medical resources. A broad application of genetic counseling to the region and social policies which reduce the founder influence could diminish the specific genetic disease burden.

Governments will probably also be influenced by the data on the general burden of genetic disease in hospitals (Table 1) when planning health care facilities in the future.

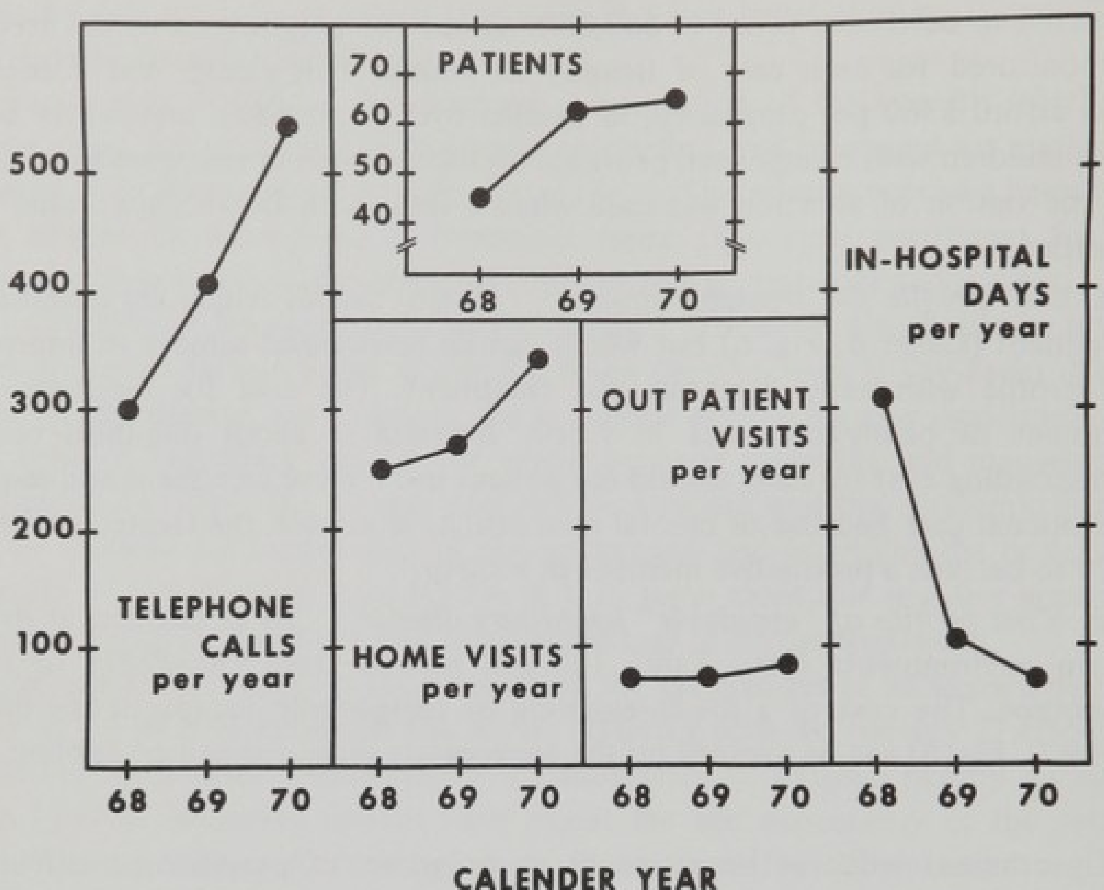

FIG. 7.–The change in patient contacts (telephone, home visiting, clinic, and hospital admission) during 3 successive years in an ambulatory regionalized program providing diagnosis, counseling, and treatment to patients with 12 different types of hereditary metabolic disease. The decline in hospitalization was related directly to the intensified ambulatory genetics program. The cost saving on bed occupancy was equivalent to half the total budget of the program. (Adapted from Clow et al., 1971.)

Attitudes of the Patient-Consumer

Screening for some forms of genetic disease has been accepted by most communities. Many of the government-sponsored programs came into existence through public law, an unlikely event if the consumer were opposed to the expenditure of tax funds in this manner. Acceptance is also encountered when the personal attitudes of the consumer are examined. For example, no parent has openly objected to the newborn screening program in Quebec. Clow et al. (1969) recorded only one parent who refused to cooperate among the 36,172 families which participated in that screening project. A very high acceptance rate is described for the many screening projects carried out by the state of Massachusetts (Levy, personal communication, 1971). We take this to mean that when the time is taken to inform about the objectives and anticipated benefits of genetic programs, the consumer will participate.

We have recently surveyed two aspects of patient acceptance in the regional

program where we are involved. A group was formed by the parents of children with four different types of hereditary metabolic disease. Meetings were held about once per month during the school year; 20 to 25 parents met regularly. Two of the present authors attended all meetings and, with the help of the parents, kept a record of the discussions and the ensuing activities, to gain some insight into the attitudes of families in the program.

In these discussions, the topics covered were problems of treatment (e.g., discipline, dietary monotony, and feeding the older patient), gene control (e.g., birth control measures and parental sterilization), handling retarded siblings, and public policy and attitudes. In addition to the apparent benefits from meeting to discuss problems of mutual concern, we noted that the group welcomed new parents into the program in a supportive way, usually by assigning an experienced couple to help the new parents to adapt to the demands of the treatment regime. The group also acted upon its discussions in an identifiable manner: one couple prepared a dietary manual and provided a translation into French; four couples sought and obtained sterilization from a gynecologist; four couples obtained Medic-Alert tags for their retarded offspring with late-diagnosed phenylketonuria; a subgroup who had children affected by the genetic disease because of late diagnosis established a preschool nursery for retarded children; the group, as a whole, challenged their government through their elected representatives and through the news media to initiate the preventive and supportive health care programs for diagnosis, counseling, and treatment of some hereditary diseases (the program described on pp. 169-179 answered their challenge).

A survey of patient-parent opinion about services for diagnosis, counseling, and treatment of hereditary disease has been carried out with 27 families in one of our regional genetic centers. Ninety-eight percent of those sent the questionnaire responded to it, a comment in itself. The following attitudes were discerned and are pertinent to this discussion:

The use of Allied Health Personnel for primary care of genetic disease was acceptable to 72% of families upon first contact; after exposure to the system 85% of the parents preferred contact with such personnel to any previous experience they knew.

Participation in research studies affected 62% of the group; none felt this was an imposition.

No parent or patient participating in a teaching clinic objected to this experience.

Half of the group were convinced that the first counseling episode was insufficient, and none would agree that they could understand their own problem and its management without some form of continuous counseling; 89% felt sufficiently informed about the progress of treatment and the significance of violations of the treatment regimen.

All but one respondent believed the genetics group was completely honest when reporting progress of the patient.

Counseling about Mendelian inheritance patterns was said to be understood by 68% of the group; 82% of those counseled about autosomal recessive inheritance gave the correct

recurrence risk when given a test question; but only 25% of these counseled for X-linked disease correctly answered their question about the recurrence risks.

Continuous counseling and the opportunity to discuss any problem about genetic disease was desirable to all, but 34% of parents were anxious to know still more than they did.

Almost all parents (96%) were very satisfied with the ambulatory approach to genetic disease control: there was no desire expressed for the traditional visit "to the doctor's office" (or clinic) in place of disease control by the techniques employing telephone, mail, and home visiting. This attitude prevailed even though half the parents were themselves responsible for obtaining the blood and urine samples of their children and 40% of these did not actually like this task.

The discovery of genetic disease adversely affected interpersonal relationships in only 4% of the families.

Forty percent of correspondents had elected to have no more children after inherited disease was discovered in their family. However, two couples decided to proceed with another pregnancy, after successful experience with treated phenylketonuric offspring; no couple with offspring harmed by the trait through late diagnosis took this option.

These attitudes reveal to us that most parents, when given the opportunity to carry out the complicated treatment regimens which neutralize the effects of the mutant allele in their offspring, will, in effect, act as substitutes for the normal allele. Common sense tells us that if we place the primary responsibility for treatment on the parents (or the patient) without adequate support, either treatment will fail, or the patient will require more time for disease management in hospital and clinic. Neither of these alternatives is as acceptable as a well-structured regional genetics program which provides in-home care for the patient.

CONCLUDING COMMENT

Genetic disease management for the few is a topic which is likely to irritate those who are concerned about much larger problems such as the feeding of the many hungry in our world. We have tried to show that the number of people in modern human society affected by genetic disease is not insignificant. We believe that the genetically afflicted of the world are as concerned for their own health as any other group of patients. Moreover, with increasing acceptance of limitation in family size within the larger web of world population control, it is more likely than not that people will be concerned about the quality of life for their offspring. The genetically blighted fetus or the congenitally malformed newborn represent forms of illness not likely to be acceptable to parents if they can be avoided. Therefore, we anticipate that society will encourage the development and application of prenatal genetic methodology (Milunsky et al., 1970), sooner rather than later, and more rather than less. The ethical issues involved in human abortion have, to some extent, been tempered with liberal social attitudes, and recent changes in abortion laws testify to this. Fletcher (1971) points out that ethical issues in genetics have a different meaning for the person who determines

right and wrong in terms of human consequences than for the ethicist who reaches his decision on a priori grounds; he asks us to consider whether fetal "rights" are in fact more important than human needs.

While the ethical and social implications of genetic disease control settle upon us, there will be additional debate about the consequences of today's activity. There are problems arising from commitments to the present generation which will affect the next. For example, what have we to offer the present generation's complement of females with phenylketonuria? When such a woman becomes pregnant, her hyperphenylalaninemia must be controlled if we wish to avoid irrevocable harm to her fetus in utero (Stevenson and Huntley, 1967). This is but one example of the apparent need for our society to make a lifetime commitment to patients with genetic disease.

What about genetic "pollution" of the species? If patients with hereditary illness are kept in health when they formerly died or were sequestered from society, and if they now marry and have children, will they augment our pool of mutant genes? Questions concerning the ultimate frequency of these genes and the rapidity with which equilibrium is achieved will demand careful attention (Kirkman, 1971). Calculations have been made by various people to show that the time to double the frequency of mutant genes can be very long (hundreds of years) or very short, depending on the inheritance pattern of the allele, the family size, and the mating patterns in practice. However, estimates of this type will be greatly modified should the art and science of carrier detection be applied widely enough to influence selection of spouse and the pattern of childbearing in families carrying mutant genes.

These issues must be focused into a meaningful commitment from society to its mutants who are, indeed, consumers with special needs (Scriver, 1971b). We have already accepted many of the challenges inherent in genetic disease control. The cost benefits of early diagnosis and treatment for many seemingly "rare" diseases have been adequately demonstrated and found acceptable. Prototype studies are likely to lead to even wider application of genetic methods in disease control. If our declarations and charters of human rights, which state implicitly that the quality of life in our mutants is equal to that of others, mean anything, and if we are all truly members of the same biological constituency, we must then all participate in the decisions which concern our heredity. Man has already shown that environmental engineering can control the expression of certain harmful genes. Consequently man can manipulate the biological destiny of some of his species now without awaiting the era of genetic engineering when he can change "bad" alleles into "good." If relatively simple methods are already available to help our mutants, our technology should be able to close the many gaps which now exist between genetic theory and the needs of our mutants. If we cannot meet this challenge, we should dismantle our present apparatus for

genetic disease control and apply the resources and savings elsewhere. The decision rests with all of us.

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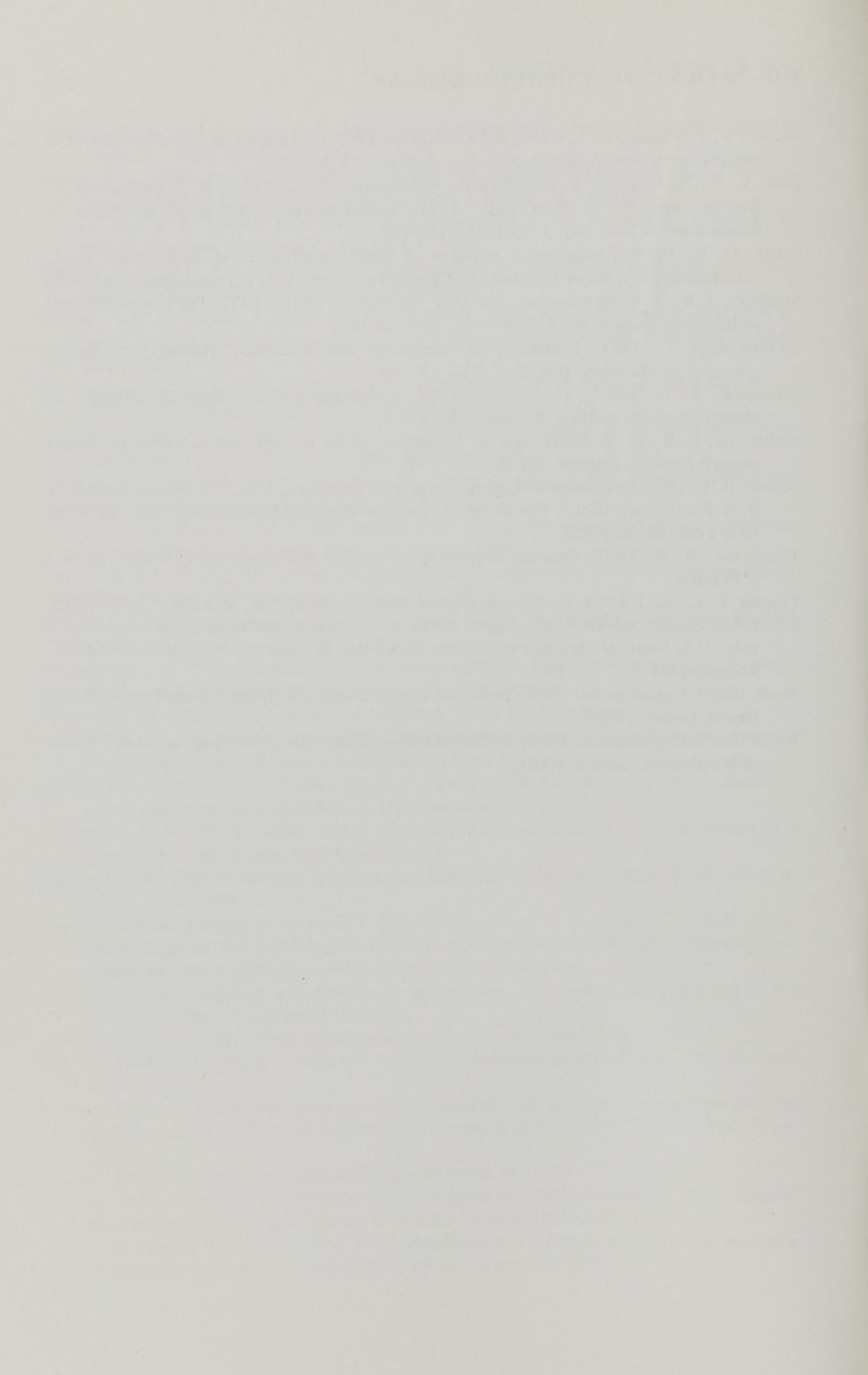

CHAPTER 5

Genetic Control of Sexual Difference

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In approaching the subject of human sexual difference, it may be useful to construct a brief outline of the processes to be reviewed [1]. Gonadal differentiation occurs from a bipotential structure, the indifferent gonad. Persistence of the outer rim or cortex leads to development of an ovary, whereas the medulla is the progenitor of the testis. In each case, remnants of the other zone may persist and provide cross-sexual phenomena of clinical importance.

The internal genitalia arise from bilateral paired primordia–either Mullerian ducts producing the Fallopian tubes, uterus, and proximal vagina, or Wolffian ducts evolving into the seminal vesicles, vasa deferentia, and epididymides. Both Mullerian and Wolffian duct primordia exist in embryos of both sexes. The external genitalia also arise from evolution of structures common to all embryos. The genital tubercle is the source of clitoris or penis; the genital folds are the origin of the labia minora of the female or, in the male, a ventral raphe whose

closure moves the urethral orifice to the tip of the phallus. Finally, the genital swellings produce either the labia majora of the female or the scrotal sacs of the male. In female development, minimal evolution of the fetal structures occurs. In the male, the fundamental processes are phallic elongation, midline fusion of the paired fold primordia, and posterior migration of the developing scrotal sacs.

The control over these processes appears to be exerted by two substances derived from the fetal testis [2]. One is a locally active substance known as an inducer or organizer; it appears to be sufficient for the process of Mullerian inhibition and necessary but not sufficient for Wolffian induction. To complete Wolffian duct induction the second fetal product is also needed; this is a steroid hormone, probably testosterone, which alone is responsible for external virilization. Thus, the weight of evidence indicates that masculinization of the fetus is the positive result of testicular secretions acting on either paired or continuously transformable primordia. Put another way, neither the ducts nor the external genitalia are inherently sexually determined, but are either potentially female, if uninfluenced, or male, if conditioned (Table 1). Since both critical influences emanate from the testis, the first question of genetic import is what directs the bipotential embryonic gonad to become a testis.

GONADAL DEVELOPMENT

Ovary

Two X chromosomes appear to be necessary for the differentiation of a normal ovary [3]. In the 45,X Turner's syndrome, instead of a true ovary the gonad is an elongate mass of connective tissue. Recently it has been noted that although the adult gonad is merely whorled connective tissue, in the early fetal Turner's gonad there are germ cells [4]. These apparently disappear, perhaps owing to the lack of development of an investing corona of follicular cells which Ohno [70] has suggested may serve a stabilizing role on the oocytes. It has also been somewhat lost sight of in discussions of the relatively normal appearance of these oocytes that (a) their total number (as opposed to number per high-power field) is very likely to be greatly diminished, and(b) the gross anatomy of the

TABLE 1.–*Roles of Male-Determining Factors, Schematized*

Process	Control substance	
	Inducer	Androgen
Mullerian inhibition	+	
Wolffian stimulation	+	+
External virilization		+

ovary is markedly abnormal and hardly conducive to a tubal relationship such as would promote fertility.

Ferguson-Smith has analyzed the structural defects of the X which inhibit ovarian differentiation, and it appears that for practical purposes all of the second X must be present [3]. Although considerable modification of the somatic defects of Turner's syndrome can be achieved by partial X presence, the recognizable X-chromosome loss compatible with ovarian function is trivial. (Ascertainment bias favoring amenorrhea as the index symptom strongly influences this statement). On the other hand, there are many instances of mosaicism compatible with prolonged periods of menstrual function including fertility [3, 5]. Though few observations of histology of the ovaries have been made, it must be inferred that a stem of 46,XX cells can have a significant influence on ovarian development. In this connection, one would like to have an idea of the proportional representation of different stems in the gonad itself, but few such studies have been done [6, 7], and their findings can hardly be considered representative of the determinants obtaining during differentiation.

Parenthetically, it is worth recognizing that a second sex chromosome plays a significant role in both somatic differentiation and survival. Ferguson-Smith originally emphasized the role of a second sex chromosome in normal somatic differentiation by comparing 45,X patients with normal females and normal males [3]. Since then several studies have documented the presence of the 45, X karyotype as one of the most common findings in human abortuses [8, 9]. This defect occurs in about 1% of human conceptions, but in only 1:4000 newborns. Since only 1 of every 40 such conceptuses survives gestation, it is clear that the absence of a second sex chromosome severely reduces viability. On the other hand, gonosomal hypoploidy is not invariably associated with complete gonadal deficiency. Both Ferguson-Smith [3] and Singh and Carr [4] have described a significant number of 45,X patients who, with or without major Turner's stigmata, have had some menstrual function. It is easy to insist that such patients all represent mosaics in which XX or XXX stems have not been detected, but in several cases numerous cells were studied–in one case (Bahner's [10]) over 1000–and all were found to be chromatin negative. It seems possible that whatever the role of the second X in stabilizing oocytes (see below), some follicular development may occur without X diploidy in occasional patients.

Testis

One of the salient lessons of human cytogenetics has been the critical role of the Y chromosome in differentiation of a testis. From comparison of the 45,X Turner's patient and the normal 46,XY male, it is clear that either the Y or some X-Y interaction is responsible for differentiation of the bipotential gonad into a testis. Several hypotheses have been proffered to explain this effect. Mittwoch

[11] suggested that the control of male vs. female gonadal development may be a special feature of the differential growth rate of cells of differing DNA content. Citing evidence that cells with more DNA replicate and divide more slowly, and, arguing that 46,XX cells have about 2% more DNA than the 46,XY, she suggested that female cells undergo mitosis more slowly. In addition to explaining the smaller size of women (this suggestion neglects endocrine influences on growth rates, incidentally), this hypothesis argues that at some point the relative smallness of the Y boosts the already greater growth rate of male embryos, perhaps by increasing their mitotic rate. Since the male gonad differentiates 5 or 6 weeks earlier than the female, this increase in mitotic rate would lead to development of a testis, and the latter's production of male hormone would then stimulate its own and subsequent male development. Supporting evidence for this was found in the observation that in vitro explants of testis grow faster than ovary. Jost commented that he doubted an effect on mitotic rate could be the sole determiner of testicular organogenesis [2].

On the basis of cytologic evidence and the occurrence of poly-X syndromes, Hamerton [12] has proposed a different interpretation which seems to the author to account for more of the experimental and clinical data. In man, the individuals with 45,X syndrome are infertile females, whereas those with 46,XY, 47,XXY, 48,XXXY, and 49,XXXXY are all males, albeit all infertile except 46,XY. Similarly, in 46,XX females there is random inactivation of the second X, unless a structurally abnormal X– is present (XXp–, XXq–, XXqi, etc.) in which case *it* is preferentially inactivated. Hamerton therefore concludes that there must be one euchromatic X per pair of haploid set of autosomes, with the second X in the female and the Y in the male being heterochromatic. He further suggests that these heterochromatic sex chromosomes serve a regulatory function. As we have seen, in the female the second X seems to be needed for a normal ovary, and its functions may be to slow the rate of follicle atresia and to regulate estrogen production. In the male, Hamerton proposed that the structural gene information for differentiation of the testis is on the X but the regulatory control is on the Y. In the presence of the latter, the processes of cortical inhibition and medullary stimulation [13] in the embryonic gonad are initiated. It is interesting that on the basis of a study of intersex disorders in domestic mammals, McFeeley et al. made a very similar proposal [14].

A review of the syndromes of familial male pseudohermaphroditism had led the author to propose a similar hypothesis. These disorders comprise a spectrum with progressively closer approximation of normal male differentiation. The minimum achievement of virilization, Mullerian suppression, is complete in testicular feminization (TF) and all the others [15]. In TF there is no Wolffian development and no external virilization, whereas in the other related disorders there is progressively more of both of these up to Reifenstein's [16] syndrome in which hypospadias, hypospermatogenesis, and gynecomastia are the only

failures of masculinization. Each of these disorders is inherited in a manner consistent with either a sex-linked or a sex-limited autosomal dominant mechanism [17-19]. The existence of very closely similar syndromes subject to the same inheritance pattern suggested that a specific genetic locus or region might be responsible for structural differentiation and functional integrity of the testis [20]. The genetic data collected on these disorders indicate this region to be on the X or, less likely, on an autosome. Since the Y is known to be a necessary factor in male development, it was inferred that it served a controlling or regulatory function.

Since the above formulation was proposed, further support for it has come from the emerging genetics of pure gonadal dysgenesis (PGD). This disorder includes the streak gonads and female ductal and external differentiation of Turner's syndrome, but lacks the short stature or somatic anomalies [21]. The gonad has the same wavy stroma usually taken to suggest ovary, but the presence of the Y suggests that, if anything, it is a would-be testis rather than an ovary. In this sense (i.e., ontogenetic rather than histopathologic), PGD is a form of male pseudohermaphroditism. Usually sporadic, cases of PGD have shown widely varying karyotypes including XX, XY, X/XX, XXp−, etc. In the present context the significant advance is the finding of several sibships in which all cases were 46,XY, the pedgrees of which were consistent with an X-linked locus (or, as in the other forms of male pseudohermaphroditism, a sex-limited autosomal dominant). Espiner et al. described 5 cases of PGD in 3 sibships in one family. All patients were 46,XY and the diagnoses were biopsy-proven [22]. This report served to exclude prior hypotheses that PGD was probably due to imperceptible deletions in the X chromosome [23]. A rather similar family reported by Sternberg et al. included 3 affected patients in 3 different sibships in the same family [24]. Although only 1 of these was proven by biopsy, the documentation seems clear and the mechanism thus supported. Other similar reports can be consulted [25, 26]. Two other reports may be significant in this context. Baron et al. [27] and Barr et al. [28] have described the occurrence of PGD and male pseudohermaphroditism in siblings; in the latter report there was also an affected sororal nephew. These cases suggest variable expressivity of a gene carried on the X and affecting testicular differentiation and function.

The presence of the Y in these patients implies that gonadal differentiation should have favored a testis. Since the transmission of the defect is through the mother, however, the defect must be on the X. And, since the defect is failure of testicular differentiation, it may be inferred that a necessary factor for medullary dominance in the bipotential gonad is located on the X.

In summary, then, several strands of evidence, when combined, suggest that the structural gene information for testicular morphogenesis is borne on the X and that the Y carries a regulatory locus which controls its expression.

The location of this center on the Y chromosome has been the subject of

some interest. The most significant patients are those with an isochromosome for the long arm of the Y (46,XYqi), presumably lacking the short arm of the Y, who are phenotypic females with gonadal dysgenesis [29, 30]. Jacobs originally suggested that the locus for the testis regulator would thus be on the area deleted in these patients, and several confirmatory reports appear to substantiate that suggestion [31]. A dosage effect of this center was alleged when a number of 47,XYY patients appeared to have elevated levels of urinary testosterone [32-34], but critical analysis of these data, as well as of other similar data reporting gonadotropin excess, has not confirmed that idea [34-36]. Another possible dosage effect of the XYY genotype may be hypogonadism or maldescent of the testis. In a review of the XYY syndrome in males in 1968, Court Brown [37] pointed to the disproportionate number who appeared to have one or both of these traits as the basis for their ascertainment. The significance of this association hinges on the frequency of the genotype in the general population and on the possibility of undetected mosaicism, particularly with a 45,X stem. Since neither is known with certainty, this possible association cannot yet be definitely interpreted.

Parenthetically here one might remark on the exciting area of fluorescent staining of human chromosomes which began with the observation that the Y has a zone of bright fluorescence when stained with quinacrine mustard [38]. This area appears to be on the distal portion of the long arm [39]. It was natural to apply this technique to the 46,XX phenotypic males with testes, of whom some 20 or more have been reported [40, 41]. These patients raised the question of whether the Y is a *necessary* factor for differentiation of a testis. In the past, several explanations have been proffered for this finding, including X-Y translocation (Ferguson-Smith [42]), Y-autosome translocation (DeGrouchy et al. [43]; Federman et al. [44]), and loss of the Y at an early stage of development of a 47,XXY zygote (de la Chapelle [45]). When the fluorescent characteristic was detected, it was hoped that it would demonstrate Y chromosome material on the putative X of such patients, but so far all reports have been negative. [46-48]. Possible explanations would be that (a) the testis determining locus (probably on the short arm) and the fluorescent locus are different, (b) the fluorescence characteristic differs when the Y is translocated, or (c) the Y is not a necessary feature for testicular differentiation. A report by Borgaonkar and Hollander describes a male patient with a small Y chromosome showing no fluorescent center [49], indicating that the heterochromatic area on the long arm of the Y is not needed for maleness, as suggested in hypothesis (a) above.

DUCT DEVELOPMENT

There is general agreement that the testis produces a substance which inhibits Mullerian duct anlage, but there has been remarkably little progress on either its

nature or its mechanism of action. A recent study showed that human fetal testis, but not ovary or adrenal, inhibited the development of Mullerian duct anlage explanted from a 14-day rat fetus [50]. In this limited study, the substance thus appears to be organ but not species specific, but whether it is a nonandrogen steroid, a protein, a polypeptide, or something else is not known.

In contrast, there has been a great deal of work on the positive influence of androgen on Wolffian development and external virilization, and that is most clearly seen in the development of knowledge of the TF syndrome. The broad nature of progress and the intensity of interest in this area warrant a separate discussion.

Testicular Feminization (*TF*)

Originally clearly defined by Morris, TF is an inherited disorder in which a chromatin-negative, 46,XY genotype is associated with bilateral testes, absence of significant Mullerian or Wolffian development, and female external genitalia. The vagina is variably deep, but ends blindly. In the so-called "complete" form there is no clitoral enlargement and, at puberty, no virilization. Breast development occurs spontaneously at puberty and the body contour feminizes, but in the absence of a uterus there is no menarche and this is the usual presenting complaint.

The endocrinology of TF is now well worked out. Although the details do not concern us in this review, the outlines show:

1. Normal urinary 17-ketosteroids.
2. Normal urinary estrogens.
3. Slightly elevated urinary gonadotropins.
4. Normal plasma follicle stimulating hormone (FSH) with mildly elevated plasma interstitial cell stimulating hormone (ICSH) (a very disturbing finding which will not be further discussed) [51].
5. Plasma testosterone (T) values in the high male range associated with lack of testosterone effect [52, 53] and
6. Impaired conversion of testosterone to its major metabolite, dihydrotestosterone (DHT) [54-56].

These last two represent the areas of major recent interest and should be discussed separately. Wilkins had shown that testosterone was inactive in these patients [57]. Southren and more recently others showed that this was so despite normal or even elevated male levels of T [51, 52]. Attention therefore focused on the intracellular metabolism of the hormone. T (in common with other steroid hormones) enters the cytosol and is initially bound to a protein receptor. There, and to an even greater extent in the nucleus, T is converted by the action of a 5α reductase to DHT. The latter is bound within the nucleus in a macromolecular complex which includes a protein receptor and seemingly attaches to the chromatin [58]. Wilson and Walker [54] showed that the

Testosterone → Dihydrotestosterone

reductase was prominent in T target tissues such as the mons pubis, but it is curiously absent from a major T target such as muscle. Since labeled DHT is the principal steroid species bound to the nuclear chromatin and since DHT is more active than T in several androgen assays [59], it was proposed that the defect in TF might be absence or failure of the reductase. This does not seem likely to be the entire or even the principal explanation, however, since (a) DHT itself is not active in TF patients [60], and (b) the reductase deficiency may be a result rather than a cause of the testosterone inactivity [61].

Several very interesting studies have been done to illuminate the TF phenomenon. Both Northcutt et al. [56] and Wilson and Walker [54] found a diminished conversion of T to DHT in target tissues which usually contain the reductase. Similarly, in a series of fascinating studies, Mauvais-Jarvis and his colleagues showed (a) that in normal males the skin metabolizes T to DHT but that in TF this does not occur; (b) that this defect can be reproduced in normal males by estrogen administration, and that patients with TF are 10 times more sensitive to this effect of estrogen; (c) that labeled T given percutaneously yields more labeled DHT than the same compound given orally or intravenously, presumably because the latter reaches the liver first and is metabolized before the skin can reduce it; and (d) that the 5α reductase of skin is negligible in TF, normal females, and hypogonadal males and is corrected in the latter two but not in TF by testosterone administration [62, 63]. To put these data together, Mauvais-Jarvis proposed that the defect in extrahepatic 5α reduction of T did not have to be a defect in the enzyme but could be the result of diminished entry of testosterone into the cell. This in turn might be due to an elevated level of the binding globulin which transports both estradiol and testosterone, with a consequent diminished amount of free testosterone available for transport into the cell. This phenomenon was validated in another series of experiments in which it was shown that the cutaneous coefficient of T 5α reduction was inversely correlated with the affinity of plasma proteins for T [63].

These observations point to the possibility of a defect in access of the steroid hormone to the nuclear chromatin, a step which is probably necessary for the initiation of transcription, which in turn appears to be the critical step in steroid hormone action [64]. There are some interesting animal data bearing on this

aspect of the binding of androgens to nuclear chromatin. The Stanley-Grumbeck male pseudohermaphrodite rat is very closely analogous to the human TF syndrome. Bullock et al. [65] showed, also in rats, that cellular uptake of T and both cytoplasmic and nuclear conversion of T to DHT were normal, but that nuclear uptake and retention of DHT were severely diminished. Similar observations have been made in animals treated with cyproterone acetate, an antiandrogen which, if given to several species of animals during gestation, converts genetic males to a phenotype remarkably similar to TF [66]. In certain species the pathology is almost identical to that in man. Walsh and Korenman have shown that this drug competitively inhibits the stimulation by both T and DHT of rat ventral prostate growth; that the conversion of T to DHT by isolated nuclear reductase was not inhibited; that after intravenous administration of T, cytoplasmic DHT appeared normally but nuclear localization of same did not occur; and that, in minces of prostate tissue incubated with T and DHT, formation of the macromolecular complex of steroid, protein, and chromatin was inhibited [67]. Thus cyproterone acetate appears to work by inhibition of attachment of the steroid to its active site. The similarity of these results and of those reported for the male pseudohermaphrodite rat suggest that this may be a significant lesion in human TF as well.

Let us summarize the metabolic findings of the TF syndrome. There is general agreement that testosterone is almost inactive and that its conversion to DHT is diminished. The latter appears to be a consequence of T inactivity, i.e., the reductase is a testosterone-induced enzyme whose absence is a reflection rather than a cause of T inactivity. The failure of T to work may be a consequence of the diminished availability of intracellular T because of its elevated plasma binding. This too may be a reflection of diminished effect of testosterone on the cells normally responsible for synthesis of the testosterone-estradiol binding globulin, whose level is normally depressed by testosterone. Finally, there is evidence for a diminished attachment of testosterone and its reduced metabolite to the nuclear chromatin, and that may well be the basic defect, as was proposed before [68].

Two important further implications of these TF studies should be mentioned. First, if it is assumed that all phenotypic consequences of the disease are due to the single mechanism of testosterone resistance, the absence of Wolffian development is proof that an androgen influence is needed, in addition to the Mullerian inhibiting factor, for male ductal development. Again, on the same assumption, testosterone is apparently the normal fetal androgen of man, something which has been much argued in the past.

There has been no significant progress on the genetic mechanism underlying TF in man. The critical question remains whether the gene is carried on the X or on an autosome. Since the affected individuals are infertile, genetic inferences must come from the demonstration of linkage with a known X-linked or

autosomal trait, and such studies have so far been uninformative. A very important bit of evidence has recently been provided by Lyon and Hawkes, who reported a syndrome very similar to TF in the mouse [69]. The genital anatomy is the same and the testis histology is similar if slightly more mature. The only evidence so far that the disease is due to testosterone resistance is the failure of testosterone to induce the renal enzyme alcohol dehydrogenase. Since Ohno has postulated that the X chromosomes of all mammals are homologous and that genes X-linked in one species are X-linked in all [70], and since there is much evidence for and none against this hypothesis, the finding of a TF syndrome which is X-linked in the mouse is of major importance. It has always seemed to the author much more likely that a sex-specific locus should be on the X than on an autosome. Indeed, in man, there is so far no evidence for a sex-determining influence on the autosomes, although several familial instances of true hermaphroditism [71] are suggestively similar to the autosomally determined polled mutation of the goat [72].

Other Forms of Male Pseudohermaphroditism

Male pseudohermaphroditism other than testicular feminization has been reported with increasing frequency lately [73, 74]. As previously suggested, the various syndromes describe a spectrum and tend to run true to form within a family. There is still rather limited information on whether the other syndromes are due to diminished production of testosterone or to various degrees of resistance to its action. Winterborn et al. described a 13½-year-old girl with incomplete TF who showed mild masculinizing changes at puberty despite plasma T and DHT levels five times normal for that age [75]. They argued that the patient had incomplete response to T because she was showing only mild masculinization despite the high androgen levels. Crawford [76] also favors the partial resistance hypothesis, in that (a) 1 TF patient raised as a male showed only minimal phallic growth and hair development despite a plasma T of 0.91, (b) another incomplete TF patient showed a diminished response to T in a balance study (the control data for this comparison were borrowed from another study and recalculated), and (c) a 2-month-old incomplete TF patient showed almost no change in external genitalia when given human chorionic gonadotropin, 600 U thrice weekly for 6 weeks, during which time a normal male would have shown marked change. In somewhat similar studies Rosenfield et al. [77] also concluded that "complete" and "incomplete" TF are respectively due to varying degrees of androgen resistance.

A possible new form of familial male pseudohermaphroditism has been proposed by Saez et al. [78], who described two chromatin-negative, 46,XY brothers who were thought to be females until they began to develop clitoromegaly and male hair distribution at puberty. The internal genitalia were male. The plasma testosterone was low for a male, but dehydroepiandrosterone

and its sulfate were elevated two to three times and androstenedione was elevated 10 times. Estrone, estradiol, and estriol were also elevated over normal male values, and after gonadectomy all these findings returned to a normal female level. At that time also, the plasma androgen levels were normal and the interconversion of T and androstenedione were normal. The authors postulate that the patients had an incomplete defect, confined to the testis, in the 17-ketosteroid reductase. The family showed 2 affected sibs, out of 17 total, of whom 10 were male; there were no other affected members. Thus the genetics suggested an autosomal recessive inheritance, but more cases will need to be studied before either the endocrine or the genetic postulates can be accepted.

EXTERNAL GENITAL DEVELOPMENT

There has been only limited progress in knowledge of congenital adrenal hyperplasia since prior reviews. There seems general agreement on four mechanisms of androgen excess in the phenotypic female—partial and complete deficiency of 21-hydroxylase, deficiency of 11-hydroxylase, and deficiency of the 3β-ol-dehydrogenase. The condition continues to illustrate that androgen, necessary for Wolffian stimulation, is not sufficient to achieve this, since these patients never have Wolffian development, presumably because of absence of the Mullerian inhibiting factor. The reported pedigrees are all consistent with an autosomal recessive mechanism, the original seeming dearth of males now being accounted for by their being underrecognized and underreported.

Several further reports have shown that deficiency of the 3β-ol enzyme is a form of male pseudohermaphroditism in the genetic male [79, 80]. A similar syndrome has been reported in which the desmolase is deficient, leading to lipid-rich adrenals, inadquate external virilization of the male [81, 82] and early death. The first case of male pseudohermaphroditism due to 17α-hydroxylase deficiency has been reported [83]. This genetic male had an incomplete vas deferens, hypospadias, a small phallus, and incomplete testicular descent, but no uterus. Since biologically active steroids require a 17-hydroxyl group, and since normal male development requires adequate testosterone, a patient who had a partial block in 17-hydroxylation might be expected to be inadequately virilized.

PUBERTY

Puberty is not ordinarily considered a subject for genetic review, but the author would like to suggest that puberty should be looked on as a set of regulatory decisions affecting both pituitary and gonadal function. These include:

(a) At the hypothalamic-hypophysial level:

i. The time of onset of increased gonadotropin production.

ii. Whether to make gonadotropin in the cyclic pattern characteristic of the female or the sustained pattern of the male.

(b) At the gonadal level:

i. How much androgen to make.

ii. How much of it to convert to estrogen.

Hypothalamic-Hypophysial Influence

Onset of Puberty. Levels of gonadotropin production are higher in adults than in infants and young children [84]. Since the structural gene information for production of gonadotropin and, probably more significant, the gonadotropin releasing factors, cannot be acquired, the delay and then onset of puberty must be a regulatory phenomenon. There is some evidence that the first event is a change in the threshold to negative feedback inhibition of the hypothalamic centers, i.e., that levels of circulating steroid hormones which in childhood are adequate to keep gonadotropin release at very low levels are unable to do so after puberty. The details of the endocrinology need not concern us here: we can assume that some type of regulation is involved and then consider the evidence that this is genetically determined.

One source of data is the timing of menarche. Table 2 is a compilation of reports relating intervals between menarches to the degree of the relationship between the individuals. It is clear that there is a striking relationship between onset of menstrual function and closeness of relationship between subjects, the monozygous/dizygous twin data being most impressive.

Further evidence for the genetic control of gonadotropic function is found in hereditary hypogonadotropic syndromes. In the male, familial hypogonadism occurs in many guises–in otherwise normal individuals [88], in association with hyposmia or anosmia [89] (Kallman's syndrome), in the Lawrence-Moon-Biedl syndrome [90], and with a variety of skeletal and midfacial defects. In most instances, transmission occurs through the mother, and the evidence generally favors an X-linked trait. In affected females hyposmia may occur but seldom a

TABLE 2.–*Correlation Between Degree of Relationship and Interval Between Menarchial Dates**

	Tisserand-Perrier [85]	Reymert and Jost [86]	Petri [87]
Identical twins	2.2 (46)	–	2.8 (51)
Fraternal twins	8.2 (39)	–	12.0 (47)
Sisters	–	10.6 (72)	12.9 (145)
Unrelated girls	–	13.9 (200)	18.6 (120)

* Time intervals are expressed in months; parentheses represent number of pairs of girls.

lack of gonadotropin. A recent report of 3 females with hyposmia and hypogonadotropism found no affected family members and stated that an authentic case of an affected female has not been found in a Kallman's syndrome family [91]; the author has however, seen one such proven case [92] and several others may exist. In any event, the male cases clearly indicate a single-gene disorder of which defective synthesis or release of gonadotropin is a part.

Rhythmicity of Gonadotropin Production. Of the second feature of pubertal gonadotropin production, its rhythmicity, almost nothing is known in man. There is clear evidence in lower animals that the brain, ultimately the regulator of gonadotropin rhythmicity, is sexualized by in utero or early post partum exposure to testosterone [93]. In other words, the indifferent state of the cerebral control of gonadotropin release is the female or cyclic pattern; when combined with a responsive ovary capable of synthesizing estrogen and progesterone, this results in periodic gonadal function. Intrauterine or neonatal exposure to testosterone changes this pattern to the acyclic male rhythm, whereas early castration of the male changes his pattern to cyclic.

There are a number of human disorders in which the result of intrauterine testosterone exposure of the female or testosterone deprivation in the male could be assessed, but so far little has been reported. Thus it would be of interest to know whether the patient with testicular feminization has cyclic gonadotropin secretion, and whether the patient with congenital adrenal hyperplasia has a tendency to the noncyclic pattern, but this information is not yet available.

Gonadal Function

Since the ovary and testis develop from a common embryonic structure, it is not surprising to find that they use analogous biosynthetic pathways. It is worth diagramming these to show that the same steroids are made by both gonads and that the dominant female hormones, estrogens, can only be made via androgens (Fig. 1).

From the genetic viewpoint which we wish to develop, it needed first to be shown that the testis can make estrogens–i.e., that the structural gene information necessary for aromatization of the A ring and removal of the C-19 angular methyl group of androstenedione and/or testosterone is present. Synthesis of estrogens from acetate, through the intermediates to testosterone, has been shown for human testis [94]. Until recently, there was little conclusive evidence that the testis does make estrogen, but even that has now been demonstrated. From indirect studies using infusions of labeled estrone and androstenedione and making certain assumptions, MacDonald et al. [95], Baird et al. [96], Longcope et al. [97], and others have suggested that the testis secretes a small amount of estrone daily. There was less convincing evidence for

ACETATE

CHOLESTEROL

PREGNENOLONE

17-OH PREGNENOLONE

DEHYDROEPIANDROSTERONE

PROGESTERONE

17-OH PROGESTERONE

ANDROSTENEDIOL

ANDROGENS:

ANDROSTENEDIONE

TESTOSTERONE

ESTROGENS:

ESTRONE

ESTRADIOL

Fig. 1.–Biosynthetic pathways of gonadal hormones.

the direct secretion of estradiol by the testis. Recently, however, two separate groups have reported spermatic vein/peripheral vein values of estradiol which show conclusively that estradiol is secreted by the human testis, albeit in small amounts [98, 99]. The converse, namely that the ovary can synthesize androgen, is clear from Fig. 1, which shows that all estrogen is obligatorily synthesized via androgen.

Genetically, then, the difference between the biosynthetic patterns of the ovary and testis should be looked upon not merely as a reflection of structural gene capacity, but as the result of two sets of regulatory decisions–how much androgen to make and how much of it to convert to estrogen. Taking the first question, the total testosterone production rate for an adult male is of the order of 6 mg per day, with over 95% produced in the testis [98]. Although there is still much disagreement on how much estradiol is secreted directly by the testis, the total amount of estradiol recoverable from the blood is only about 30 μg per day, and thus the amount made directly from testosterone in the testis is certainly no more than 15 μg per day, or about 0.25% of the total production.

Turning it around, the ovarian secretion of testosterone in the normal adult female is about 150 μg per day [100] and of estradiol about 150 μg* [95, 101]. If we assume, for simplicity, 100% efficiency of yield, the 150 μg of estradiol means an additional ovarian "production" of 150 μg of testosterone. Thus the total production of testosterone by the ovary can be roughly estimated as 300 μg per day, of which 50% is taken further to estradiol.† In comparing values for the testis and ovary (and admitting the large inaccuracies underlying these data), we approach the figures given in Table 3. Similar calculations could be made for androstenedione and estrone.

Since these regulatory choices are a direct consequence of the pathway of differentiation of the gonad, and since the latter is determined by the presence of a Y or a second X chromosome, the second sex chromosome can be seen as responsible for the determination of certain regulatory controls of enzymatic function.

TABLE 3.–*Conversion of Testosterone to Estradiol in Testis and Ovary*

	Testosterone produced per day (μg)	% converted to estradiol
Testis	6000	0.25
Ovary	300	50.0

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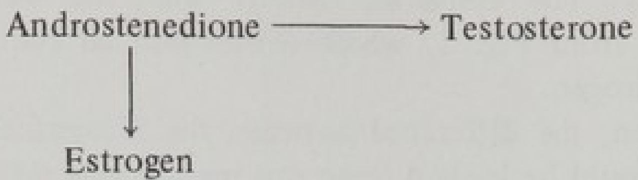
* This figure varies from 0.1-0.5 mg per day during the menstrual cycle.

† Since the molecular weight of testosterone is greater than that of estradiol, the figures as weights are not, strictly speaking, correct. They are given as approximations to illustrate the point.

In another context Short [102] has proposed that the point of decision for this regulation is in the fate of androstenedione, that in the male this precursor is metabolized to testosterone but in the female to estrogen:



The question of whether the Y directly controls this, however, is moot, because (a) in the XX goat with the polled gene, testosterone is secreted by the testis [72] and (b) the XX freemartin gonad secretes testosterone [102]. Thus Short concludes that testosterone is secreted by persisting medullary tissue regardless of the genotype but that sex chromosomes normally determine the presence or absence of medullary tissue and thus the fate of sex steroid precursors. In the normal process, then, genetic control is exerted on germ cells and on the steroid-synthesizing somatic cells of the genital ridge. The rest of the sexual differentiation of the body is not determined genetically but by the action of steroid hormones.

CONCLUSION

Control of sexual differentiation and development in man appears to represent a remarkably economical disposition of energy. Normal embryos have the potential for either male or female development. A genetic message determines the initial pathway of gonadal differentiation, and the results of that choice appear to control subsequent events. Although the outlines of these processes are becoming clear, much remains to be learned about the cellular events which implement these dictates.

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CHAPTER 6

The Familial Dyslipoproteinemias*

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*Abbreviations used in the text of this review: HDL, high-density or alpha lipoproteins. LDL, low-density or beta lipoproteins. VLDL, very-low-density lipoproteins, which technically includes pre-beta or endogenous VLDL and chylomicrons. The latter contain mostly exogenous or dietary glycerides. Generally, VLDL is used to denote pre-beta lipoproteins, i.e., endogenous VLDL, and the term chylomicrons is reserved for exogenous VLDL only. PHLA, postheparin lipolytic activity, a measure of the enzyme lipoprotein lipase in plasma or tissue. LCAT, lecithin-cholesterol acyl transferase.

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For over a century we have known that blood contains substantial amounts of fat, yet only recently have medical scientists begun to define closely the relationship between blood fats and disease in man. A number of well-planned epidemiologic studies have shown that risk of coronary heart disease is related directly, though not exclusively, to plasma cholesterol and, perhaps, to plasma glyceride concentrations (Brown et al., 1965; Werkö, 1971; Kannel et al., 1971). Although many other factors, including blood pressure, body weight, glucose tolerance, and exercise, are also related, plasma cholesterol concentration has been the single best predictor of coronary disease risk (Kannel et al., 1971).

The plasma lipids include cholesterol, triglycerides, phospholipids, hydrocarbons, and fat-soluble vitamins (Fredrickson and Lees, 1966). Plasma concentrations of cholesterol and triglycerides have been measured most often. The values have not been symmetrically distributed about population means (Schaefer, 1964; Thomas et al., 1964). In fact, some studies have shown two or three modes of plasma triglyceride concentration (Schaefer, 1964). These data suggest that the population includes subjects with genetically determined hyperlipidemia.

Plasma lipids are largely insoluble in water. They do not occur in the free state in plasma, but rather in combination with protein as *lipoproteins*. Plasma lipid measurements represent the sum of the lipid contents of the several lipoprotein classes. It is therefore more accurate to speak of disorders of lipoprotein metabolism than of lipid metabolism. Likewise, the inherited disorders of plasma lipid concentrations should be studied via the inheritance of and the control mechanisms for the concentration of plasma lipoproteins rather than of the lipids.

In this review, the structure and function of the lipoproteins are considered briefly and the diseases of lipoprotein metabolism are discussed in detail. Among these diseases are three rare but well-defined hypolipoproteinemias from which much of our knowledge of the control of lipoprotein concentrations has been learned. We will not attempt to discuss in detail methods of lipoprotein analysis or treatment of the hyperlipoproteinemias, as this information appears elsewhere (Lees and Hatch, 1963; Hatch and Lees, 1968; Lees and Wilson, 1971).

THE PLASMA LIPOPROTEINS

Chemical analysis, ultracentrifugation, electrophoresis, and other methods reveal five major lipid-carrying protein classes in human plasma.

The Circulating Lipoproteins

Free Fatty Acids (FFA)–Albumin. Plasma albumin transports free or unesterified fatty acids (FFA) through plasma (Fredrickson and Gordon, 1958; Jeanrenaud, 1961; Steinberg, 1963). FFA, which provide most of the body's energy during fasting, are produced by lipolysis of adipose tissue triglycerides. FFA are bound to albumin by noncovalent forces upon entering the plasma (Goodman, 1958). They are removed by skeletal and cardiac muscle, liver, and other major sites of utilization. Because the FFA concentration in plasma is low (300-700 meq/liter), and because they are measured as fat-soluble acids rather than as proteins (Dole, 1956), they are not usually considered among the lipoproteins. Their short half-life in plasma (usually 2-3 min) makes FFA concentration labile and very responsive to nervous and hormonal stimuli.

High-Density Lipoproteins (HDL, or Alpha Lipoproteins). The plasma HDL float in the ultracentrifuge between density 1.063 and 1.21 g/ml (Havel et al., 1955). They are quite heterogeneous. The major components have alpha-1 mobility by electrophoresis and share a common protein moiety, which apparently consists of two or three major polypeptides of molecular weight about 15,000 each (Shore and Shore, 1959). The two major HDL classes, HDL_2 and HDL_3, can be partially separated in the analytical ultracentrifuge by their densities. The HDL_2 has density 1.063 to 1.12, and HDL_3 has density 1.12 to 1.21. They contain about 50% protein, 30% phospholipids, and small amounts of cholesterol esters and triglycerides (Hatch and Lees 1968). The alpha lipoproteins carry most of the plasma phospholipids.

Two other lipoproteins have been identified in this density fraction. HDL_1 is a lipoprotein of density about 1.063 which immunologically seems to be LDL or a mixture of LDL and HDL. The LP antigen (Schultz et al., 1968; von Wiegant et al., 1968; Sodhi, 1969) is a lipoprotein (density 1.063 to 1.12) which reacts as LDL immunologically and has pre-beta mobility on electrophoresis. It has been called "sinking pre-beta" (Rider et al., 1970). This lipoprotein, originally considered an allotypic antigen, has been identified in about 10 to 30% of normal and hyperlipidemic human plasma.

Low-Density Lipoproteins (LDL, or Beta Lipoproteins). LDL normally seems comparatively homogeneous. It floats in the ultracentrifuge between densities 1.006 and 1.063 g/ml and usually forms a single sharp peak in the analytical ultracentrifuge. Apparently, LDL has a uniform, well-characterized protein moiety, strongly antigenic and with several subunits (Shore and Shore 1967; Margolis and Langdon, 1966a,b,c; Kane et al., 1970). LDL is the major carrier of cholesterol in human plasma. By weight, the molecule contains about 22% protein and nearly 50% cholesterol and its esters.

Very-Low-Density Lipoproteins (VLDL, or Pre-Beta Lipoproteins). The lipoproteins of density less than that of plasma (1.006) are classified as VLDL.

This class spans a broad size and density spectrum between plasma density and the density of triglyceride itself (about 0.9 g/ml).

Two important subclasses of the VLDL are the pre-beta, or "endogenous," lipoproteins and the chylomicrons, or dietary fat particles (Lees and Fredrickson, 1965b). The pre-beta lipoproteins are synthesized primarily in the liver, and perhaps also in the intestine, either from adipose tissue fatty acids, or by direct synthesis from nonlipid precursors. They are the major glyceride-bearing lipoproteins in the plasma during fasting, and they consist of 50 to 90% triglycerides by weight. VLDL size is directly related to the particles' relative glyceride contents, and density is inversely so related. Normal human subjects in the postabsorptive state have very low pre-beta-lipoprotein concentrations consistent with their low plasma glyceride concentrations.

Chylomicrons appear in plasma after a fatty meal (Gage, 1920; Gage and Fish, 1924) as very large fat particles which will float in the ultracentrifuge at plasma density. They contain small amounts of protein, cholesterol, and phospholipids (Dole and Hamlin, 1962; Zilversmit, 1965) but consist mostly of triglycerides which are identical to and presumably derived directly from dietary fat. Long-chain dietary fatty acids enter the body almost entirely via chylomicrons, which are apparently synthesized only by the intestinal mucosa (and only during fat absorption), are secreted into the intestinal lacteals, and then enter the venous blood via the thoracic duct.

Lipoprotein Allotypes

Many studies of allotypy in human plasma lipoproteins have been conducted, most of which have been epidemiologic and have used immunodiffusion assays. A number of allotypic systems for LDL have been described (Blumberg et al., 1962; Berg, 1965; Bütler, 1967). The alleged allelic genes appear unrelated to lipoprotein concentrations or to the diseases of lipoprotein metabolism. Presumably, therefore, they affect the structure of the beta-lipoprotein molecule in regions which are not important in its metabolism. Recently, two LDL allotypic systems were reassessed. The Australia antigen, first described by Blumberg et al. (1965), apparently is either the virus of serum hepatitis or a closely related antigen (Prince, 1968). The LP antigen, also originally considered an allotypic LDL antigen, was identified as a distinct and separate new lipoprotein class, as mentioned. These findings, and the tremendous heterogeneity of human plasma lipoproteins, suggest that other apparently allotypic systems may, in fact, be previously unappreciated lipoprotein antigens of metabolic importance or associated with structurally abnormal lipoproteins.

Lipoprotein Metabolism

A normal subject's plasma after a 12- to 15-hour fast contains HDL and LDL and small amounts of VLDL (Fig. 1). The lipids of these three lipoproteins

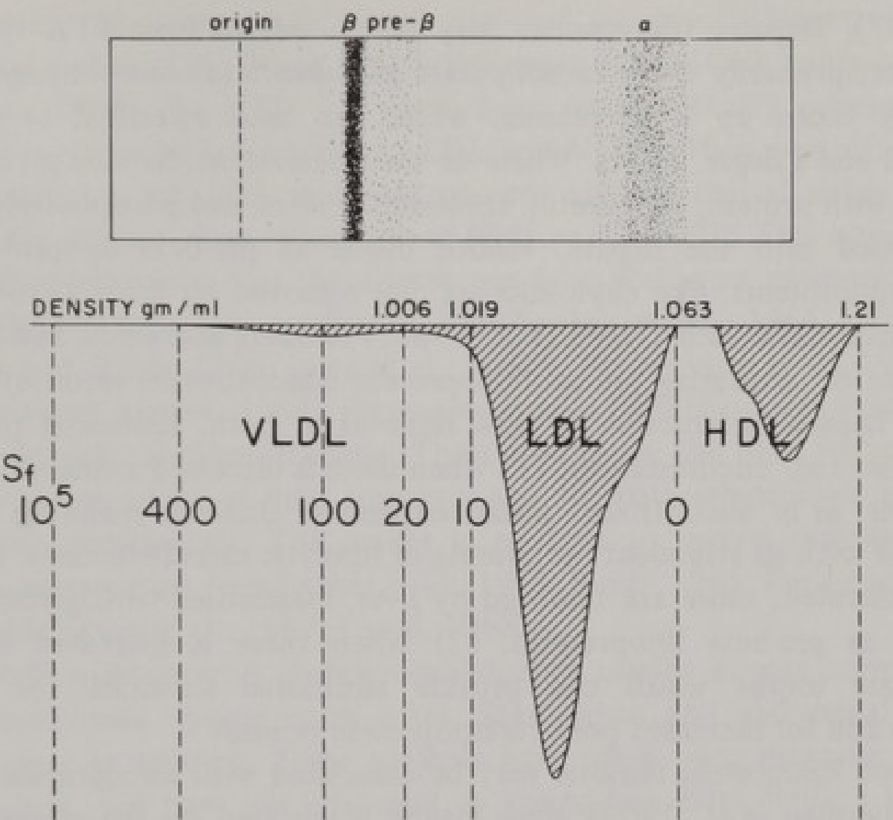

FIG. 1.–The normal plasma lipoprotein pattern in the fasting state. At the top of this and the following figures is a representation of a lipoprotein electrophoresis on filter paper (Lees and Hatch, 1963). Below it is a typical analytical ultracentrifuge pattern. HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, Very-low-density lipoproteins.

represent the normal plasma concentrations of cholesterol of about 200 mg/dl (mg per deciliter, i.e., per 100 ml of plasma) and of glycerides of about 75 mg/dl.

Chylomicrons appear in plasma only after a fat-containing meal. Three to four hours after fat ingestion, plasma glycerides rise to a value not exceeding 300 mg% and the plasma becomes slightly turbid due to its chylomicron content. Because their removal rate from plasma is very rapid, chylomicrons disappear from blood 6 to 9 hours after a fat-containing meal. Chylomicron removal appears to be a two-stage process (Redgrave, 1970). First, glycerides are stripped from chylomicrons and are hydrolized by lipoprotein lipase. The fatty acids so formed are removed by adipose tissue, liver, skeletal and cardiac muscle, and many other tissues of the body. Then the cholesterol-rich "remnants" are removed by the liver.

Pre-beta lipoproteins are produced primarily in the liver, although during fasting, the gut may produce some as well (Baxter, 1966: Ockner et al., 1969). The quantitative contribution of the gut to circulating plasma pre-beta-lipoprotein levels is still unknown. The rate of pre-beta-lipoprotein secretion by liver is probably regulated by the liver's function of triglyceride export (Jones

et al., 1967). Hepatic triglycerides may be assembled from FFA synthesized within liver, primarily from carbohydrate precursors (*de novo* lipogenesis), or from FFA taken up from plasma, which are then esterified to glycerides (Schonfeld and Pfleger, 1971). Whatever their derivation, hepatic glycerides are combined with protein, cholesterol, cholesterol esters, and phospholipids and are then secreted into the hepatic venous blood as pre-beta lipoproteins. The pre-beta lipoproteins, like chylomicrons, are removed by most tissues, largely adipose tissue and muscle (Havel et al., 1962; Friedberg and Estes, 1964).

Increases in circulating pre-beta-lipoprotein concentration result either from enhanced hepatic production or slow removal or both. Enhanced production occurs under two circumstances: (1) When there is increased availability of FFA to the liver, as in uncontrolled insulin-dependent diabetes mellitus, or during acute stress with its attendent outpouring of lipolytic catecholamines. As plasma FFA are elevated, more are removed by liver, reesterified to triglycerides, and resecreted as pre-beta lipoproteins. (2) When there is increased caloric or carbohydrate intake which can provide additional substrate for *de novo* lipogenesis and for increased pre-beta-lipoprotein release.

Decreased lipoprotein removal may be associated with uncontrolled diabetes mellitus (Bierman et al., 1966); since insulin is required for the maintenance of normal levels of lipoprotein lipase, the enzyme presumably is responsible for the removal of both pre-beta lipoproteins and chylomicrons from plasma (Kessler, 1963). Each of these mechanisms has been implicated in the pathogenesis of hyperlipidemia in man.

The factors which control the plasma concentrations of LDL and HDL are still unclear. Whether LDL are synthesized *de novo* by the liver or whether they arise entirely from VLDL degradation is still under discussion (Bragdon et al., 1956; Langer et al., 1970). The major protein moiety of LDL occurs in VLDL in sufficient quantity to account for most or all of LDL in plasma. The sites of LDL removal from plasma are also unknown. The half-life of LDL in plasma has been measured and apparently is about 3 days (Volwiler et al., 1955; Gitlin et al., 1958; Lees and Ahrens, 1969).

HDL are probably made in the liver, at least partially, since their apoproteins in VLDL cannot account for all of the HDL in plasma. Their half-life in plasma is about 4 days (Furman et al., 1964). Their sites of removal are also unknown.

THE INHERITED HYPOLIPOPROTEINEMIAS

Abetalipoproteinemia

In 1950 two siblings were described with a syndrome of malabsorption, demyelinating neuropathy, and peculiar thorny-appearing red blood cells (Bassen and Kornzweig, 1950). Additional cases of this peculiar disease were reported in

the following 10 years from several parts of the world (Singer et al., 1952; Jampel and Falls, 1958; Druez, 1959; Friedman et al., 1960; Lamy et al., 1960; Mier et al., 1960). In 1960 Salt et al. demonstrated that a patient with this syndrome completely lacked plasma LDL and VLDL. They postulated that the inability to form the LDL molecule was primary and that all other manifestations of the disease were related to it (Salt et al., 1960). This hypothesis and their further postulate that the disease was inherited as an autosomal recessive trait were subsequently confirmed (Isselbacher et al., 1964; Wolff, 1965; Farquhar and Ways, 1966; Levy et al., 1966a; Fredrickson et al., 1967; Lloyd, 1968; Lees and Ahrens, 1969). Extensive studies have been performed on many patients with abetalipoproteinemia. These studies are the basis for much of our present understanding of lipid transport and the pathophysiologic mechanisms of hyperlipoproteinemia. The reader is referred to several reviews for more extensive information (Isselbacher et al., 1964; Wolff 1965; Farquhar and Ways, 1966; Fredrickson et al., 1967; Lloyd, 1968).

Although patients with abetalipoproteinemia usually appear normal at birth, clinical symptoms appear soon after. Steatorrhea and abdominal distension develop early in infancy. These patients have often been diagnosed as having celiac disease, but have not improved on gluten-free diets. When a low-fat diet has been selected either by the physician on an experimental basis or by the child himself, the gastrointestinal symptoms regress considerably.

Signs of neurologic disturbance have usually appeared at about age 5. Muscular weakness, ataxia, and, in many patients, retinal degeneration are usually evident by adolescence. Death has occurred from progressive muscular weakness and paralysis, from intercurrent infection, or from congestive heart failure due to myocardial degeneration (Dische and Porro, 1970).

Laboratory findings in this disease are striking. Plasma cholesterol concentration is usually below 50 mg/dl and almost never above 100 mg/dl. Plasma triglycerides are low or undetectable. Lipoprotein analyses reveal the complete absence of LDL and VLDL (Fig. 2). Even by sensitive immunochemical techniques, detection of any trace of LDL in affected patients has been impossible (Levy et al., 1966a).

Abetalipoproteinemic patients are completely unable to transport long-chain fatty acids from the gut, i.e., to incorporate them into chylomicrons (Ways et al., 1967). They are also unable to transfer glycerides out of the liver via the pre-beta lipoproteins (Ahrens et al., 1965; Levy et al., 1966a; Lees and Ahrens, 1969). Some evidence suggests that small amounts of long-chain glyceride fatty acids may be absorbed directly as FFA via the portal vein, a route ordinarily reserved in man and animals for the absorption of relatively short-chain fatty acids (Kiyasu et al., 1952; Ahrens et al., 1965; Kayden and Medick, 1969). These observations indicate that normal transport of lipids from both gut and the liver into the plasma depends on the presence of the LDL peptides. They

TABLE 1.–*Abetalipoproteinemia: Clinical and Laboratory Features*

References	Patient number	Sex	Age at detection, yr	Parental consanguinity	Neu-ropathy	Reti-nopathy	Acantho-cytosis	Serum choles-terol, mg/dl
Bassen and Kornzweig (1950)	1	F	18	1st cousins	+	+	+	52
Singer et al. (1952)	2	M	13	2nd cousins	+	+	+	37
Jampel and Falls (1958)								
Kornzweig and Bassen (1957)	3	M	11	1st cousins	+	+	+	72
Druez (1959)	4	F	30	Grandparents were 1st cousins	+	+	+	60
Salt et al. (1960)	5	F	<2	None	–	–	+	22
Friedman et al. (1960)	6	M	36	None	+	+	+	42
Lamy et al. (1960, 1961)	7	M	5	Half-sibs	+	–	+	25
Mabry et al. (1960)	8	F	13		+	–	+	32-50
Mier et al. (1960)	9	M	18	None	+	+	+	46
Wolff and Bauman (1961)	10	M	5	None	+	+	+	40
Ways and Simon (1964)								
Ways et al. (1963)	11	M	<3		+	–	+	34
Schwartz et al. (1963)	12	M	9	4th cousins	+	–	+	35
Lamy et al. (1963)	13	F	<5	None	+	+	+	36
Sobrevilla et al. (1964)	14	F	15	None	+	+	+	52
Becroft et al. (1965)	15	M	<1	2nd cousins	+	–	+	23
Forsyth et al. (1965)	16	M	<2	1st cousins	+	+	+	29
Farquhar and Ways (1966)	17	F	<8	None	–	–	+	
Hooghwinkel and Bruyn (1966)	18	M	<1	Cousins	+	–	+	38
Ways et al. (1967)	19	M	7	None	+	–	+	37
Bach et al. (1967)	20	M	<1	None	+	+	+	<50
Jones and Ways (1967)	21		7				+	44
Lees (1967)	22	M	<1	None	–	–	+	25-40
Dische and Porro (1970)	23	M	7	None	+	–	+	18
Crain (pers. commun.)	24	M	9	None	+	–	+	74-155
Ibid.	25	F	12	None	+	–	+	41-60

– not detected; + present; blank spaces indicate information is not available.

TABLE 2.–*Tangier Disease: Clinical and Laboratory Features**

References	Patient number	Sex	Age at detection, yr	Lympho-megaly	Hepato- or spleno-megaly	Neuro-pathy	Total choles-terol, mg/dl	Phos-pho-lipid, mg/dl	Tri-glycer-ide, mg/dl	Cholesterol in		
										VLDL,† mg/dl	LDL,‡ mg/dl	HDL,§ mg/dl
Fredrickson (1966)	1	F	6	–	–	–	84	11	205	53	31	<1
Ibid.	2	M	5	+	+	–	93	127	225	35	56	2
Ibid.	3	F	8	–	–	–	107	137	224	14	92	1
Ibid.	4	F	12	–	+	–	67	96	151	12	54	1
Ibid.	5	M	45	+	+	–	30	68	142	19	11	1
Ibid.	6	M	48	–	–	–	50	114	213	33	13	1
Engel et al. (1967)	7	F	16	–	+	+	68	70	138	15	51	2
Ibid.	8	F	24	–	–	+	84	118	164	24	59	1
Kocen et al. (1967)	9	M	37	–	+	+	47	110	332	34	8	8
Kummer et al. (1968)	10	M	40	–	+	+	68	27	122			2
Huth et al. (1970)	11	F	3	+	–	–	61-77	70-110	114-196			7

*– not detected; + present; blank space indicates information is not available.
† VLDL, very-low-density lipoproteins.
‡ LDL, low-density lipoproteins.
§ HDL, high-density lipoproteins.

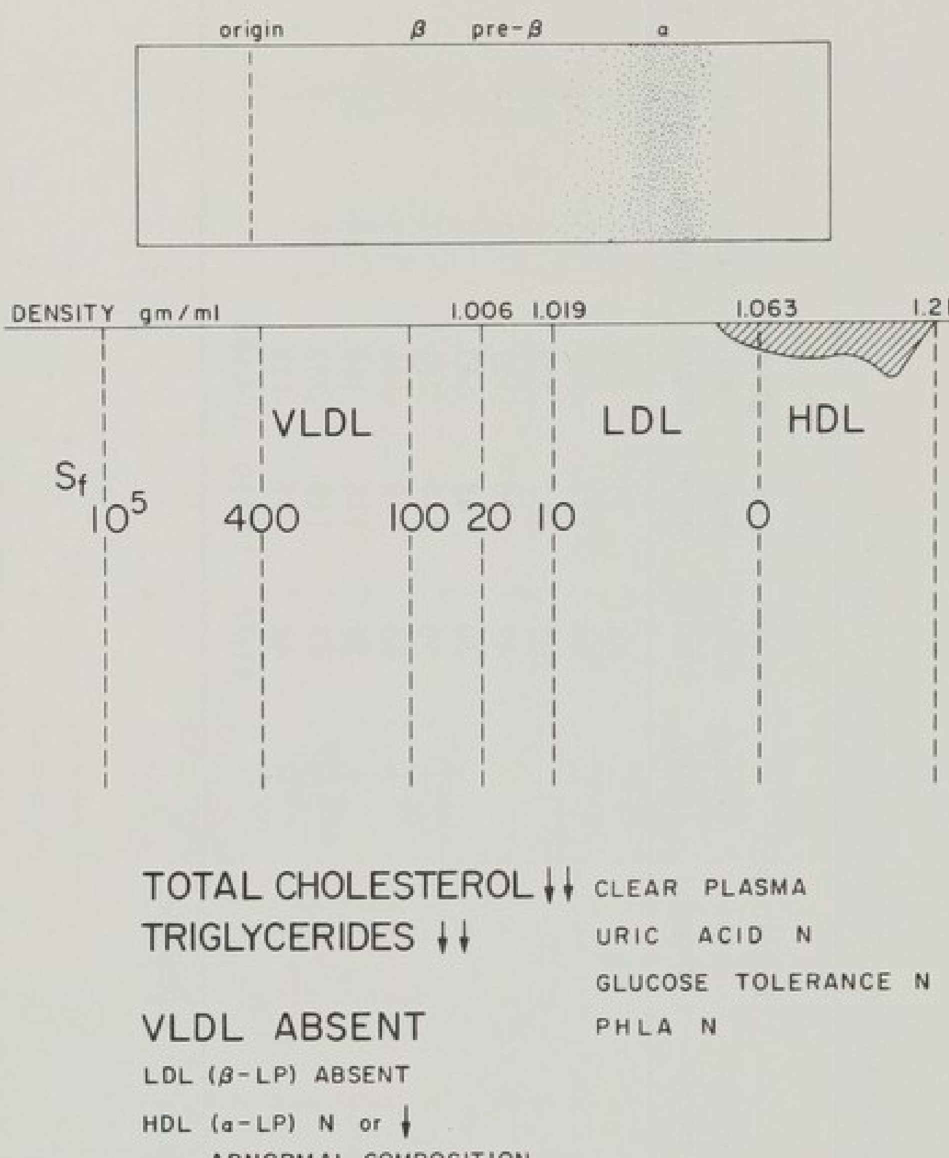

FIG. 2.–Laboratory findings in abetalipoproteinemia. The lipoprotein patterns are as in Fig. 1. At the bottom of this and the subsequent figures are listed the major laboratory findings for each disease. PHLA, postheparin lipolytic activity.

further suggest that a major function of those apolipoproteins is the transport of glycerides from liver and gut.

HDL in abetalipoproteinemia are also abnormal. The phospholipids of HDL are abnormal in their relative proportions and fatty acid composition (Jones and Ways, 1967), and their density is therefore less than normal, so that they often float in the LDL range (Levy et al., 1966a).

Another important finding in this disease is the presence of characteristic thorny or spiny erythrocytes called acanthocytes (Ways et al., 1963; Ways and Simon, 1964; Simon and Ways, 1964). In the absence of severe liver dysfunction, acanthocytosis is pathognomonic of abetalipoproteinemia. The multiple neurologic findings were discussed earlier. The reader is referred to

other reviews for clinical details (Isselbacher et al., 1964; Lamy et al., 1960, 1961, 1963; Wolff, 1965; Lloyd, 1968; Farquhar and Ways, 1966).

Clinical data relative to the inheritance of abetalipoproteinemia are summarized in Table 1. Neither parent has been involved with the disease; there has been, however, frequent sibling involvement. The data are consistent with autosomal recessive inheritance.

Many patients with diminished, but detectable, circulating LDL have been reported (Kuo and Basset, 1962; Mars et al., 1969; Van Buchem et al., 1966a, b). Acanthocytosis and neurologic degeneration have also been reported in the absence of lipoprotein abnormalities (Estes et al., 1967; Critchley et al., 1968; Levine et al., 1968). These diseases are even rarer than abetalipoproteinemia and are apparently unrelated to the latter genetically or metabolically.

Tangier Disease

Tangier disease is a rare familial disease named after a small island in the Chesapeake Bay, the home of the first patients. The disease is characterized by plasma HDL deficiency. It was first reported in 1961 in a 5-year-old boy whose tonsils were strikingly enlarged and yellow (Fredrickson et al., 1961). The patient and an older sister were studied at the Clinical Center of the National Institutes of Health where the tonsils were described as infiltrated by cholesterol esters. The patients' disorder was initially diagnosed as Niemann-Pick disease, a tissue lipidosis, until the results of routine lipoprotein analysis showed almost no HDL present in the plasma. At least 11 patients have been described in the medical literature, 8 of whom were sibling pairs (Table 2).

Clinically, the disease has been quite variable. The 6 patients described by Fredrickson et al. had a relatively benign disease, diagnosed in 2 in the fifth decade of life. All the patients had large tonsils of distinctive orange or yellow color, identified either by history or on physical examination. Two of these patients had enlarged lymph nodes and hepatosplenomegaly (Fredrickson, 1966). One had pancytopenia which necessitated splenectomy (Hoffman and Fredrickson, 1965). One patient died at 48 of presumed coronary artery disease; unfortunately, postmortem examination was not performed. Four of the five patients subsequently described (Kocen et al., 1967; Engel et al., 1967; Kummer et al., 1968; Huth et al., 1970) had severe neurologic disorders, manifested clinically as peripheral polyneuropathy with loss of sensation, weakness, and muscle wasting. In the patients studied, cholesterol esters were deposited in the reticuloendothelial cells of nearly every tissue examined, including the corneas, the arterial wall, the skin (Waldorf et al., 1967), and the rectal mucosa.

The *laboratory findings* in Tangier disease are striking. Total plasma cholesterol varied from 30 to 107 mg/dl; values for most patients were below 100 mg/dl. Plasma triglycerides were very frequently, albeit mildly, elevated. Lipoprotein analyses show a great diminution of HDL (Fig. 3). HDL cholesterol

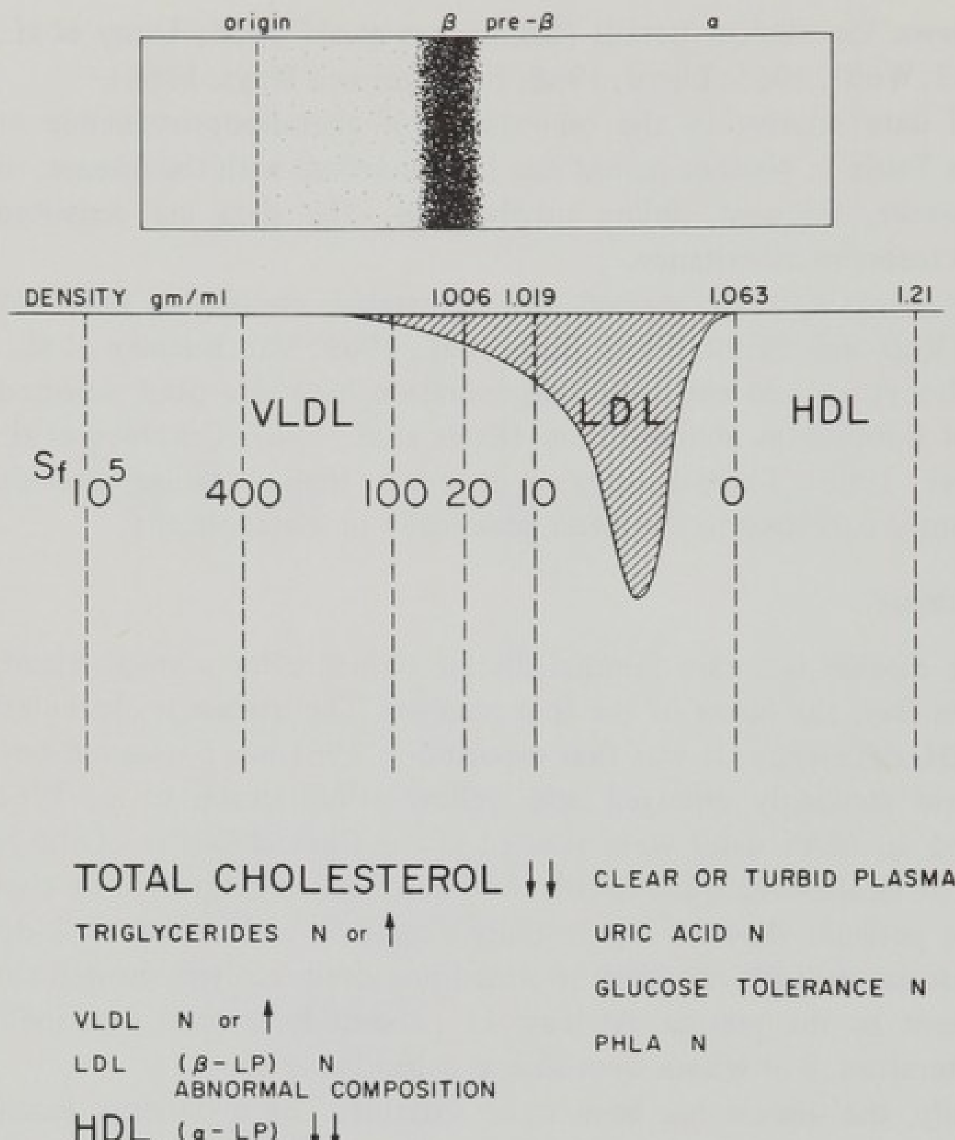

FIG. 3.–Laboratory findings in Tangier disease, arranged as in Fig. 2.

is reduced to 5 to 15% of normal, although HDL phospholipids and triglycerides may be somewhat less diminished (Kocen et al., 1967). Immunologically, HDL are reduced to a few percent of the normal concentration (Fredrickson, 1966). The small amount of HDL is immunologically slightly different from normal, but the protein moieties contained in it seem chemically identical to the normal. The LDL in Tangier disease contain increased amounts of phospholipids (Fredrickson, 1966; Kocen et al., 1967) and the VLDL have beta rather than pre-beta electrophoretic mobility (Levy et al., 1966b). The lipids of VLDL are also of unusual composition (Shacklady et al., 1968).

The mild hyperlipidemia of patients with Tangier disease is exacerbated by high-carbohydrate diets. Plasma VLDL are increased as in normal subjects; however, the VLDL is always of beta rather than pre-beta electrophoretic mobility. Thus, patients with Tangier disease export hepatic triglyceride via abnormal VLDL. Chylomicron formation and fat tolerance are grossly normal.

We conclude from these studies that HDL or their protein moieties are more

important in the removal of glycerides than in facilitating their entrance into the plasma. Pathologic studies have shown cholesterol ester accumulation in nearly every tissue examined. Thus, HDL also seem necessary for cholesterol transport from tissue.

Tangier disease has provided an unusual opportunity for genetic studies, since the population of the island home of the first 2 patients is only about 900 (Fredrickson, 1964). Careful survey of the patients, their relatives, other inhabitants of Tangier Island, and normal controls (Fredrickson, 1964) showed that the disease itself was inherited as a Mendelian recessive trait, but that heterozygotes had approximately half-normal HDL concentrations. The inheritance of a given plasma HDL concentration appeared controlled by a pair of alleles. In this gene for Tangier disease, one abnormal allele produced no apparent disease but a chemically diminished HDL concentration, whereas two abnormal alleles produced the clinical disease. Interestingly enough, some patients heterozygous for the disease have also been hyperlipemic, have had labile plasma HDL concentrations (Fredrickson, 1966), have exhibited marked carbohydrate induction of lipemia (Levy et al., 1966b), and have had normal HDL as well as an immunologically distinct HDL circulating simultaneously in the plasma.

The exact nature of the biochemical defect is still unknown. There is no convincing evidence for an abnormality in the HDL protein moiety and no other possible causes are known.

Familial Plasma Cholesterol Ester Deficiency (*Norum-Gjone Disease*)

A 33-year-old Norwegian woman entered the University Hospital in Oslo in 1966 with anemia, proteinuria, hyperlipemia, and diffuse gray corneal opacities. Although most routine clinical laboratory tests gave normal results, her plasma was found to be virtually lacking in esterified cholesterol, which usually comprises about 65 to 75% of total plasma cholesterol (Norum and Gjone, 1967ab; Norum et al., 1971b). Two of the patient's sisters exhibited similar clinical and laboratory findings, and 2 other sisters were healthy. Further studies showed, that the patient's plasma lacked the enzyme lecithin-cholesterol acyl transferase (LCAT), an enzyme known for more than 30 years previously (Sperry, 1935; Glomset, 1968), but whose importance in lipid and lipoprotein metabolism was not known.

The disease is clinically similar to Tangier disease (Torsvik et al., 1968; Norum et al., 1971b). Common *clinical features* include infiltration of the cornea with many minute gray particles distributed in all layers, denser toward the limbus where it resembles arcus corneae (Gjone and Bergaust, 1969), anemia, and proteinuria, along with red blood cells and casts in the urine (Table 3). The reader is referred to the references for complete clinical details.

TABLE 3.–*Norum's Disease (Familial LCAT Deficiency): Clinical and Laboratory Features**

Patient no.†	Sex	Age at detection, yr	Corneal opacity	Anemia	Protein-uria	Hyper-lipemia	LCAT‡
1	F	19	+	+	+	–	–
2	F	31	+	+	+	+	–
3	F	33	+	+	+	+	–
4	F	47	+	+	+	+	+
5	M	Post-mortem§	+	+	+	+	–
6	M	35	+	+	+	+	–
7	F	42	+	+	+	+	–

* – not detected; + present. Table from Norum et al., 1971b.
† Patients 1-3 are siblings, as are patients 4-5 and patients 6-7.
‡ LCAT, lecithin-cholesterol acyl transferase.
§ Died of uremia.

Laboratory findings include an increase in plasma total cholesterol; values were elevated in 5 of the 6 patients reported. Virtually all of this was free cholesterol, with cholesterol esters comprising only 5 to 10%. Plasma triglycerides and phospholipids were slightly to greatly elevated. Phospholipid distribution in plasma was grossly abnormal. Lecithin, which usually constitutes about 60% of the total plasma phospholipids, represented 82 to 86% of the total in all the known patients. On paper lipoprotein electrophoresis only an LDL band appeared. HDL and pre-beta lipoproteins were not detectable (Norum and Gjone, 1967b). Ultracentrifugal studies have demonstrated, as with Tangier disease, LDL of beta mobility (Fig. 4). Immunologically and morphologically recognizable HDL are present, but their lipid content is too low to stain well for lipid; furthermore, they migrate in the alpha-2 rather than the alpha-1 position (Norum et al., 1971a,b; Forte et al., 1971). The erythrocytes of the 3 diseased sisters contained twice the normal cholesterol. Although the total phospholipid concentration was normal, lecithin was proportionately increased (Gjone et al., 1968).

The role of lecithin-cholesterol acyl transferase (LCAT) in lipid transport has been reviewed by Glomset (1968). The presence of a cholesterol-esterifying enzyme in plasma was first suspected by Sperry (1935), who noted that, with incubation of serum at 37°, the free cholesterol present gradually decreased and the esterified cholesterol increased, up to about 48 hours of incubation, when all the free cholesterol had been exhausted. The observations were carried much further by Glomset et al. (Glomset, 1962; Glomset and Wright, 1964; Glomset et al., 1966; Glomset, 1968; Akanuma and Glomset, 1968), who named the enzyme and identified many of its characteristics. LCAT apparently binds

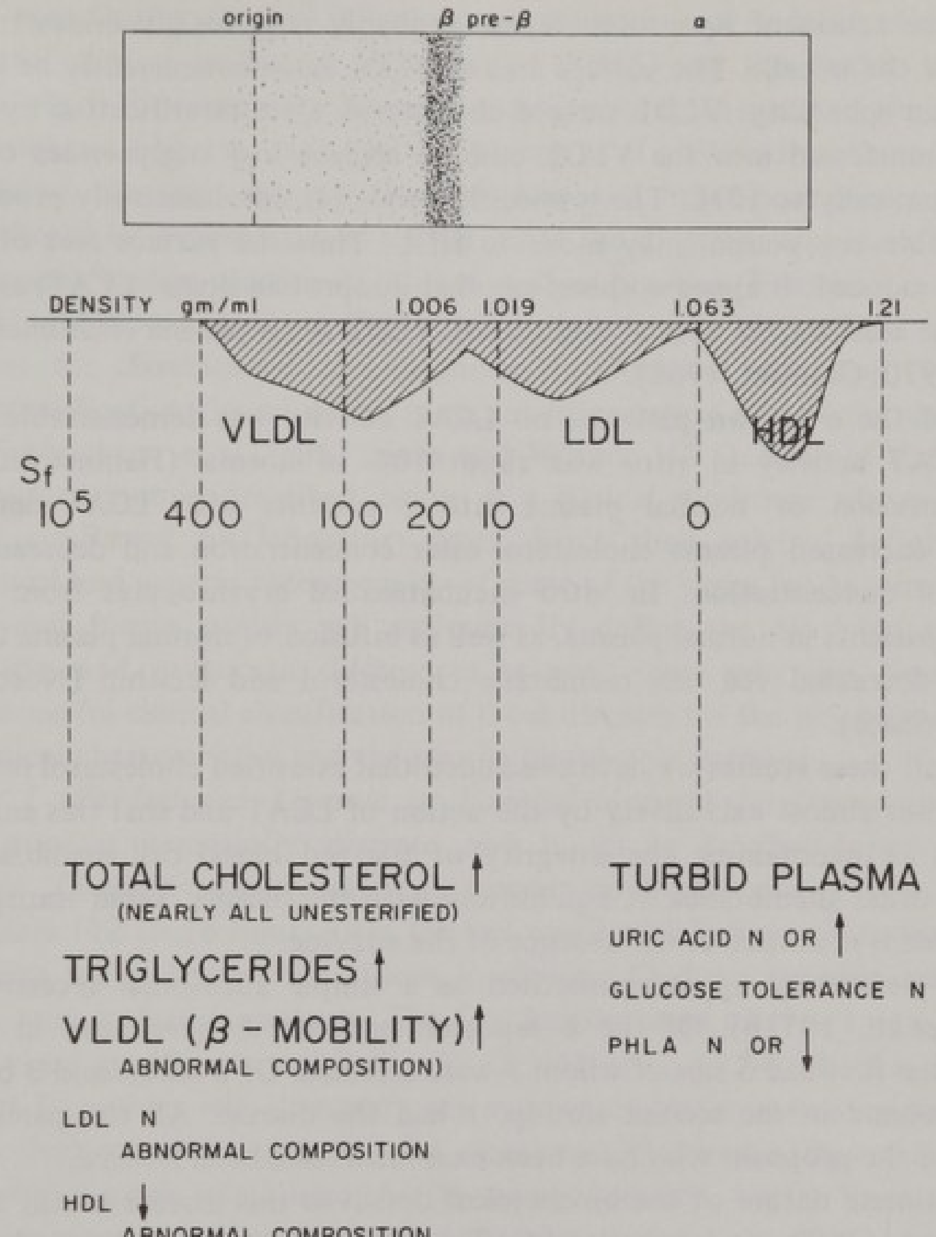

FIG. 4.–Laboratory findings in LCAT deficiency, arranged as in Fig. 2. LCAT, lecithin-cholesterol acyl transferase.

strongly to HDL, its primary substrate, and catalyzes the transfer of an acyl group from the beta carbon of lecithin (phosphatidyl choline) to free cholesterol, with the formation of a cholesterol ester molecule and a molecule of lysolecithin. Its action could account for the presence of all of the esterified cholesterol in plasma.

Glomset further suggested that the enzyme's activity, coupled with exchange of glycerides between glyceride-rich VLDL and the phospholipid-rich HDL (Nichols and Smith, 1965), could account for many of the changes which occur during normal metabolism of human lipoproteins. VLDL are spherical micelles with triglycerides and cholesterol esters in their hydrophobic interior. Phospholipids and cholesterol coat the surface of the VLDL. During VLDL removal from

plasma, the action of lipoprotein lipase probably removes glycerides from the interior of the micelle. The surface area of VLDL must concurrently be reduced to maintain sphericity. VLDL surface cholesterol, after esterification by LCAT, may be transferred into the VLDL core to replace lost triglycerides or move nonenzymatically to HDL. The lysolecithin which is simultaneously produced, a water-soluble compound, may move to HDL. Thus the surface area of VLDL would be reduced. It appears, therefore, that lipoprotein lipase, LCAT, and HDL may all be essential to normal removal of VLDL from plasma (Schumaker and Adams, 1970; Glomset, 1968).

In 5 of the 6 known patients, no LCAT activity was demonstrable. In the sixth, LCAT activity in vitro was about 10% of normal (Hamnström et al., 1969). Infusion of normal plasma into 2 patients with LCAT deficiency gradually increased plasma cholesterol ester concentration and decreased free cholesterol concentration. In vitro incubation of erythrocytes from LCAT-deficient patients in normal plasma, as well as infusion of normal plasma into the patients, decreased red cell membrane cholesterol and lecithin (Norum and Gjone, 1968a,b).

From all these studies we have concluded that esterified cholesterol in human plasma arises almost exclusively by the action of LCAT and that this enzyme is important in maintaining the integrity of the red blood cell membrane and probably other membranes in equilibrium with the plasma. Lipid transport by lipoproteins is abnormal in the absence of the enzyme.

LCAT deficiency appears inherited as a simple autosomal recessive trait (Norum et al., 1971b). Of the 6 reported patients, 5 have been in only 2 sibships; the first had 5 sibs of whom 3 were affected. Of 2 sisters and 3 brothers of the proband in the second sibship, 1 had the disease. All the parents and children of the propositi who have been examined have been normal.

The ultimate nature of the biochemical defect in this disease is still unclear, although an inability to synthesize LCAT or the synthesis of a miscoded enzyme could be responsible. The possible existence of LCAT-refractory lipoproteins in these patients has not been completely excluded. Many of the clinical features of the disease such as cataracts, anemia, and proteinuria are not wholly explainable in terms of LCAT deficiency alone.

THE HYPERLIPOPROTEINEMIAS

Primary Familial Hyperlipoproteinemias

Familial hyperlipoproteinemia is much more common than hypolipoproteinemia; some of the former syndromes have been recognized for many years. Bürger and Grütz (1932) described type I hyperlipoproteinemia nearly 40 years ago, and Addison and Gull (1851) described type II hyperlipoproteinemia over a

century ago. During the last 20 years, milestones in our understanding include the careful clinical studies of Thannhauser (1958) and the studies of Ahrens et al., in which the clinical, nutritional, and biochemical aspects of these diseases were correlated (Ahrens et al., 1957; Ahrens et al., 1961; Knittle and Ahrens, 1964). Most recently, the synthesis of these and other data into a clinically useful scheme by Fredrickson et al. has made the diagnosis and classification of these diseases readily possible in routine medical practice (Fredrickson and Lees, 1965; Fredrickson and Lees, 1966; Fredrickson et al., 1967). That scheme is used here for classification and diagnosis (Fredrickson and Lees, 1965) with certain modifications based on more recent information (Fredrickson and Lees, 1966: Fredrickson et al., 1967; Lees and Wilson, 1971). Each of the types of hyperlipoproteinemia described below is a clinical syndrome whose diagnosis may be confirmed by laboratory tests. Since their original definition, the biochemical and genetic heterogeneity of some of the types has become obvious. Current and future studies will undoubtedly define the biochemical, pathophysiologic, and prognostic differences between these subtypes. Nevertheless, the most useful clinical classification of these diseases for the present is based on their clinical characteristics and the plasma lipoprotein patterns.

Type I (Fat-Induced Lipemia or Familial Hyperchylomicronemia). This disease appears genetically discrete and is, in its familial form, clinically consistent. Characteristically, there is massive chylomicronemia (Fig. 5) in the fasting state (12 to 16 hours after the last meal) with low concentrations of the other three major lipoprotein classes in plasma. Chylomicronemia may persist for days even in fasting patients. The first known cases of type I were reported over 30 years ago (Bürger and Grütz, 1932; Holt et al., 1939). Havel and Gordon described 3 brothers who demonstrated massive chylomicronemia, gross retardation of chylomicron clearance, and decreased lipoprotein lipase activity in plasma after heparin administration (Havel and Gordon, 1960). Known cases of this disease were summarized in 1966 (Fredrickson and Lees, 1966). An updated record of known patients, both published and unpublished, appears in Table 4.

Typically, affected neonates seem quite normal, but *clinical symptoms* appear soon after. Hepatosplenomegaly is noted within a few days or weeks after birth. Eruptive xanthomas appear over the extensor surfaces or diffusely. Frequently, affected infants have numerous colicky episodes sometimes accompanied by fever, nausea, vomiting, or diarrhea. Lipemia retinalis may be noted by a physician. Whole blood may have a "hot chocolate" or "cream of tomato soup" appearance. The plasma is milky-white and creamy. The plasma glyceride level is greatly elevated; the plasma cholesterol relatively less so.

If the infant is by chance or choice on a low-fat diet, he is often well for a long time. The child himself may associate the intake of dietary fat with periodic abdominal pain or eruptive xanthomas. Often a diagnosis is not made until the patient reaches adolescence or adulthood. Pregnancy usually severely exacer-

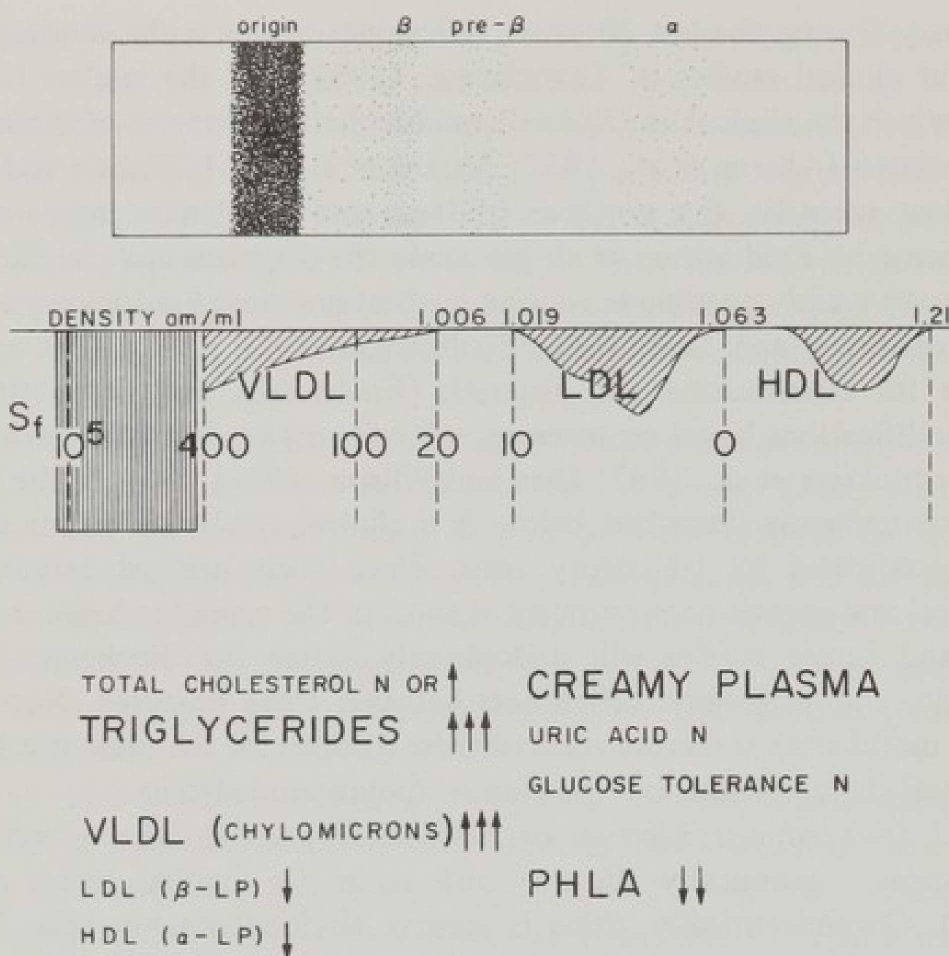

FIG. 5.–Laboratory findings in type I hyperlipoproteinemia, arranged as in Fig. 2.

bates the disease. Certain aspects of the *clinical diagnosis* warrant further discussion in detail: hepatosplenomegaly, abdominal pain, eruptive xanthomas, lipemia retinalis, and atherosclerosis.

Hepatosplenomegaly is nearly universal in type I. Enlargement of the liver and spleen is related to the amount of fat ingested. Even with massive hepatosplenomegaly, organ size decreases perceptibly within 24 to 48 hours of beginning a fat-free diet, and often returns to near normal within a few days. However, there generally remains slight to moderate enlargement, presumably from scarring due to multiple instances of fatty infiltration.

Episodic abdominal pain is characteristic of any severe lipemia, but is experienced most often in types I and V. Pain is not related to any given plasma lipid level nor is it attributable to a rapid rise or fall in plasma lipid concentrations. It is usually of gradual rather than abrupt onset and may be associated with mild or profound anorexia, nausea, vomiting, diarrhea, fever, and leukocytosis. Pain may be diffuse or localized. It may suggest splenic infarction or hepatic capsular stretching. The pain may mimic pancreatitis or may produce bona fide secondary pancreatitis (Frederickson and Lees, 1966). At laparotomy, which has been performed in a few patients, only erythema of the serosal surfaces and small amounts of clear or milky peritoneal fluid have been found.

Rarely, xanthoma-like lesions may occur on the serosa. Hyperlipemia must always enter into the differential diagnosis of obscure or atypical abdominal pain.

In severe hyperlipemia, especially in type I, eruptive xanthomas are common. Characteristically, these lesions are 2- to 3-mm papules on an erythematous base. The centers are creamy, yellow, tan, or orange and may release a little milky fluid when opened. The lesions may be single or grouped; they may enlarge and become confluent. Chronic lesions may be as large as a centimeter in diameter. Eruptive xanthomas form primarily on extensor surfaces: the back, the shoulders, or the buttocks. In severe cases they may be diffusely distributed. Although eruptive xanthomas occur only in severe lipemia, like abdominal pain they cannot be associated closely with a particular level of plasma glycerides, nor with a given rate of rise or fall in plasma lipids.

Lipemia retinalis is also characteristic in severe hyperlipemia. When the concentration of large light-refracting, glyceride-bearing lipoproteins (i.e., chylomicrons and/or pre-beta lipoproteins) reaches high levels, normal plasma streaming forms a milky or creamy layer at the periphery of the fundic vessels. Both the arteries and veins appear milky-white through an ophthalmoscope. The whole fundus may have a milky cast in severe lipemia. The appearance of the fundus can thus be used to approximate the plasma glyceride concentration.

Atherosclerosis has not been prominent in patients with type I disease, even when plasma cholesterol and glyceride concentrations have been elevated for many years. No patient with the disease has had any of the clinical signs or complications of atherosclerosis, even though some have survived into their fourth and fifth decades with sustained plasma cholesterol concentrations of 300 mg per 100 ml or more.

The *laboratory findings* in type I hyperproteinemia are striking. The hallmark of this disease is the extremely creamy plasma of untreated patients. Whole blood, as noted, may have a chocolate hue because of the extreme lipemia. Plasma glycerides are elevated, usually to values above 2000 mg/dl. Whole plasma cholesterol varies from normal to 1000 mg/dl or higher. The ratio of plasma glycerides to cholesterol is usually greater than 8 to 10. The lipoprotein pattern is diagnostic (Fig. 5). The only major lipoprotein band seen on paper electrophoresis is the dense chylomicron band. LDL and HDL concentrations are low and may not be visible on the paper electrophoretogram. The concentration of pre-beta lipoproteins is usually normal or only slightly elevated unless the patient has been on a low-fat, high-carbohydrate diet. Since the chylomicrons are the only lipoprotein in appreciable concentration, plasma refrigerated in a test tube overnight will form a dense chylomicron layer at the top and a clear subnatant. This simple finding is diagnostic of type I.

Other analytical methods for lipoproteins give results comparable to paper electrophoresis. Among those which have been used are PVP gradient floccula-

TABLE 4.–*Type I: Clinical and Laboratory Features* *

References	Patient no.	Sex	Age at detection, yr	Eruptive xanthomas	Hepato-spleno-megaly	TC,† mg/dl	TG,‡ mg/dl	PHLA §
Bürger and Grütz (1932)	1	M	1	+	+			
Abegg (1937)	2	F	1	+	+	686	6565	
Bernstein et al. (1939)	3	M	4	+	+	1059	5430	
Holt et al. (1939)	4	F	4	+	+	505	4198	Low
Goodman et al. (1940)	5	M	1	+	+	379	3000	
Levy (1946)	6	M	8	–	+	292	2606	Low
Ibid.	7	F	4	–	+	162	1044	Low
Ibid.	8	F	<1		+	146	1118	Low
Hopgood (1948)	9	M	<9	+	+	250	3202	
Kennedy and Colett (1949)	10	M	2	+	+	372	3567	
Poulson (1950)	11	F	5	–	+	320	3565	
Ibid.	12	M	4	–	+	582	3408	
Crocker (1951)	13	F	<1	+	–	740	4650	
Bruton and Kantor (1951)	14	M	2	+	+	320	2321	
Hetzel (1951)	15	M	23	–	+	130	4150	
Klatskin and Gordon (1952)	16	M	18	+	–	426	1126	
Gaskins et al. (1953)	17	M	10	–	+	234	2014	Low
Ibid.	18	M	8	–	+	238	2946	Low
Ibid.	19	M	2	–	+	363	1361	Low
Binkowska-Fellmen (1958)	20	F	<1	+	+	270	17000	
Rausen and Adlersberg (1961)	21	F	<1	+	+	500		

Ibid.	22	M	1	+	+	198	1217	
Ibid.	23	F	8	–	+	285	3530	
Bialkin et al. (1962)	24	M	2	+	+	460	3270	
Ibid.	25	M	2	+	+	420	3375	
Ibid.	26	F	<1	+	+	395	3978	
Kuo and Bassett (1963)	27	F	16	+	+	265	2475	
Fredrickson et al. (1963)	28	M	<1	+	+	357	4345	Normal
Ibid.	29	F	17	–	+	302	3371	Low
Knittle and Ahrens (1964)	30	M	<1	+	+	778	15000	Low
Ibid.	31	F	8	–	–	–	5500	Low
Fredrickson and Lees (1966)	32	F	<1	–	+	–	–	Low
Ibid.	33	F	4	–	–	–	–	Low
Ibid.	34	F	1	+	+	–	–	Low
Nevin and Slack (1968)	35	F	4	+		354	3390	
Ibid.	36	F	3	+			1560	
Ibid.	37	M	11				3375	
Braunsteiner et al. (1968)	38	M	3	+	+	750	4000	Low
Maldonado et al. (1970)	39	F	7	–	+	140	1020	Low
Ibid.	40	M	9	–	+	100	683	Low
Ibid.	41	F	<2	–	+	85	209	Low
This report	42	F	36	–	–	340	2900	
Ibid.	43	M	21	–	–	348	3990	

* – not detected; + present; blank space indicates information is not available.

† TC, total serum cholesterol.

‡ TG, serum triglyceride.

§ PHLA, postheparin lipolytic activity.

tion (Gordis, 1962; Bierman et al., 1962), starch or Pevikon block electrophoresis (Bierman et al., 1962), analytical ultracentrifugation (Fredrickson et al., 1968), and agarose gel electrophoresis (Noble, 1968).

A low post-heparin lipolytic activity (PHLA) is characteristic of type I (Fredrickson et al., 1963). After intravenous administration of heparin the lipolytic activity of the plasma is no greater than 20 to 25% of normal in type I patients (Havel and Gordon, 1960), whereas PHLA is usually normal or only minimally depressed in other types. Most plasma postheparin glyceridolysis is due to the enzyme lipoprotein lipase, although postheparin phospholipase and monoglyceridase activities have been found (Vogel and Zieve, 1964; Vogel and Bierman, 1967; Greten et al., 1969). The availability of lipoprotein lipase appears to be the rate-limiting step in removal of chylomicron glycerides from plasma. This removal apparently occurs by stepwise degradation of chylomicrons at the endothelial surface of the capillaries in adipose tissue. The liberated fatty acids are assimilated by adipocytes, and chylomicron remnants are removed by the liver (Redgrave, 1970). An inherited lipoprotein lipase deficiency apparently is the pathogenetic basis of familial type I disease. Not only are PHLA assays subnormal, but adipose tissue lipoprotein lipase is deficient as well (Harlan et al. 1967).

The molecular defect in type I may be in the enzyme itself (Steiner, 1968). The lipoprotein lipase in diseased plasma reportedly differs from normal PHLA in the effects of various inhibitors and its apparent K_m (Bradford et al., 1968). These data suggest a genetically determined structural abnormality in lipoprotein lipase (Bradford et al., 1968). Recent purification of lipoprotein lipase from man (Fielding, 1969) should permit direct validation of this hypothesis. The reader is referred elsewhere for reviews of lipoprotein lipase and lipoprotein removal (Fredrickson and Lees, 1966).

Both fasting plasma glucose concentrations and glucose tolerance are normal in type I patients unless intercurrent pancreatitis has resulted in secondary diabetes mellitus. Tests which are not affected by the high turbidity of lactescent plasma are usually normal. Grossly abnormal values for most photometric determinations may be reported unless plasma blanks are run. Since lipoprotein lipase-dependent chylomicron removal is apparently deficient in adipose tissue, heart, and skeletal muscle, chylomicrons are removed by the reticuloendothelial elements of the liver and spleen, resulting in hepatosplenomegaly. Histologic examinations reveal large fat-filled histiocytes in the liver, spleen, and bone marrow. These cells are indistinguishable from those of most other lipid storage diseases and may lead to the mistaken diagnosis of Niemann-Pick or Tay-Sachs disease (Tanaka et al., 1963).

The concept of fat induction of lipemia in type I was introduced in 1961 (Ahrens et al., 1961). Although fat induction also occurs in type V, it is the hallmark of type I disease. Fat-containing diets severely exacerbate lipemia, whereas

diets low in or free of fat markedly decrease plasma glyceride concentrations and ameliorate symptoms. Glyceride levels usually fall with fat-free diets from the several thousands to the several hundreds with a concomitant decrease in liver and spleen size and remission of abdominal pain. Type I patients remain mildly lipemic even on fat-free diets, both because of their inability to remove VLDL from plasma and because of carbohydrate induction due to the increased proportion of carbohydrate calories required by the low-fat diet. Long-chain fat may be replaced by medium-chain triglycerides (MCT), which are absorbed via the portal vein as FFA (Furman et al., 1965) with similar clinical improvement.

Evidence concerning the inheritance of type I appears elsewhere (Fredrickson and Lees, 1966; Fredrickson et al., 1967). The data are consistent with autosomal recessive inheritance of the clinical disease. Both parents and children of affected subjects are clinically well but siblings are often affected. When plasma PHLA or adipose tissue lipoprotein lipase is measured in patients and their relatives, a somewhat different pattern emerges. Many of the asymptomatic relatives have low PHLA activity, although not generally so low as the affected relatives (Fredrickson and Lees, 1966), which suggests that the heterozygous relatives have a partial defect in lipoprotein lipase activity.

The frequency of the gene for the disease in various human populations seems quite low, since the number of cases reported in the medical literature is small and penetrance appears high. In addition to patients with clear familial disease, many patients have been reported with intermittent or sporadic fat-induced lipemia. One patient of the authors had a single episode of classical fat-induced lipemia which did not recur despite large intake of dietary fat. Modifying factors such as obesity or alcoholism may reveal latent lipoprotein lipase deficiency.

Acquired fat-induced lipemia may be seen in uncontrolled diabetes (Bierman et al., 1966), pancreatitis (Kessler et al., 1963), and acute alcoholic intoxication (Isselbacher and Greenberger, 1964). Low PHLA in congenital or acquired fat-induced lipemias has been attributed to circulating enzyme inhibitors (Kessler et al., 1963) or a defective lipoprotein lipase (Bradford et al., 1968), to a failure of lipoprotein lipase synthesis in the presence of insulin lack (Pav and Wenkeova, 1960; Kessler, 1963; Schnatz and Williams, 1963; Bagdade et al., 1968a) or to in vivo heparin binding by abnormal globulins (Glueck et al., 1969).

Type II (*Familial Hyperbetalipoproteinemia*). Type II hyperlipoproteinemia is characterized by an increase in plasma beta (and frequently pre-beta) lipoproteins in the fasting state. LDL in Type I are normal chemically, physically, and immunologically. Typical in this common disease is high risk of morbidity and mortality from atherosclerosis, particularly coronary heart disease. Many patients have familial disease, apparently inherited as an autosomal dominant trait. There are many others, as will be discussed, with hyperbetalipoproteinemia of apparently polygenic or environmental origin.

Clinical symptoms are often absent in type II patients. The first sign of disease may be a fatal atherosclerotic complication in some patients, whereas lipid deposits in the skin, tendons, or eyes may bring others to medical attention. Typically, type II patients are healthy in early life. Frequently, a fatal coronary event in a parent may mar their childhood. As young adults, they may notice grayish opacification at the corneal limbus, the arcus corneae. The knuckles develop nodular irregularities and patients have described themselves as "double jointed." Achilles tendons become thickened and uncomfortable. Shoes may become tight and chafe. During early adulthood, there can be recurrent episodes of a peculiar asymmetric polyarthritis with swelling of knees and ankles but without any residual deformity. Xanthelasma (yellow or golden plaques in the skin around the eyes) are often removed surgically, only to recur.

A typical type II patient has the first of several heart attacks in his late 30's or early 40's, only after which is his blood examined. Treatment of the hypercholesterolemia and modest hyperglyceridemia with diet or drugs is then attempted for the first time. In the past, treatment has not generally lowered plasma lipids to levels even approaching normal nor have xanthomas regressed appreciably. Ultimately, the patient dies of coronary artery disease 20 years before his peers.

Xanthomatosis (the appearance of fat-containing lesions in the skin and subcutaneous tissues) was the first recognized sign of hyperlipoproteinemia (Rayer, 1835; Addison and Gull, 1851). Chauffard and La Roche (1910) first described the relationship between LDL concentration and tendinous xanthomas or xanthelasma. This relationship was firmly established by the thorough studies of the Donner Laboratory Group at Berkeley (Gofman et al., 1954).

Tendon xanthomas, although they develop most often in type II, also occur in type III disease. Characteristically, these form in the extensor tendons, i.e., the Achilles tendon, the finger extensors, the patellar tendons, and the extensor tendons of the toes as they cross the plantar fascia. The normal Achilles tendon is smooth, thin in the middle, and flaring at its origin and insertion. When xanthoma first infiltrates it, the Achilles tendon becomes slightly irregular in the middle, then becomes thickened and irregular. Later, it is as thick in the middle as at the ends and rather irregular throughout. When fully developed, Achilles tendon xanthomas are hard and irregular and encroach on the calf muscles (Fig. 6). Xanthomas in the finger extensors usually begin immediately proximal to the knuckles and can be felt to move when the fingers are flexed and extended. The process may advance to gross distortion of the knuckles and distortion of the interphalangeal joints (Fig. 7). At first, patellar tendon xanthomas cause slight enlargement of the tibial tubercles. Later they enlarge enough to suggest the development of bone tumors. The lesions are radiolucent since they are comprised of cholesterol-containing xanthomatous tissue. Xanthomas of the toe flexors usually occur in the plantar fascia of the transverse metatarsal arch and may interfere with weight bearing.

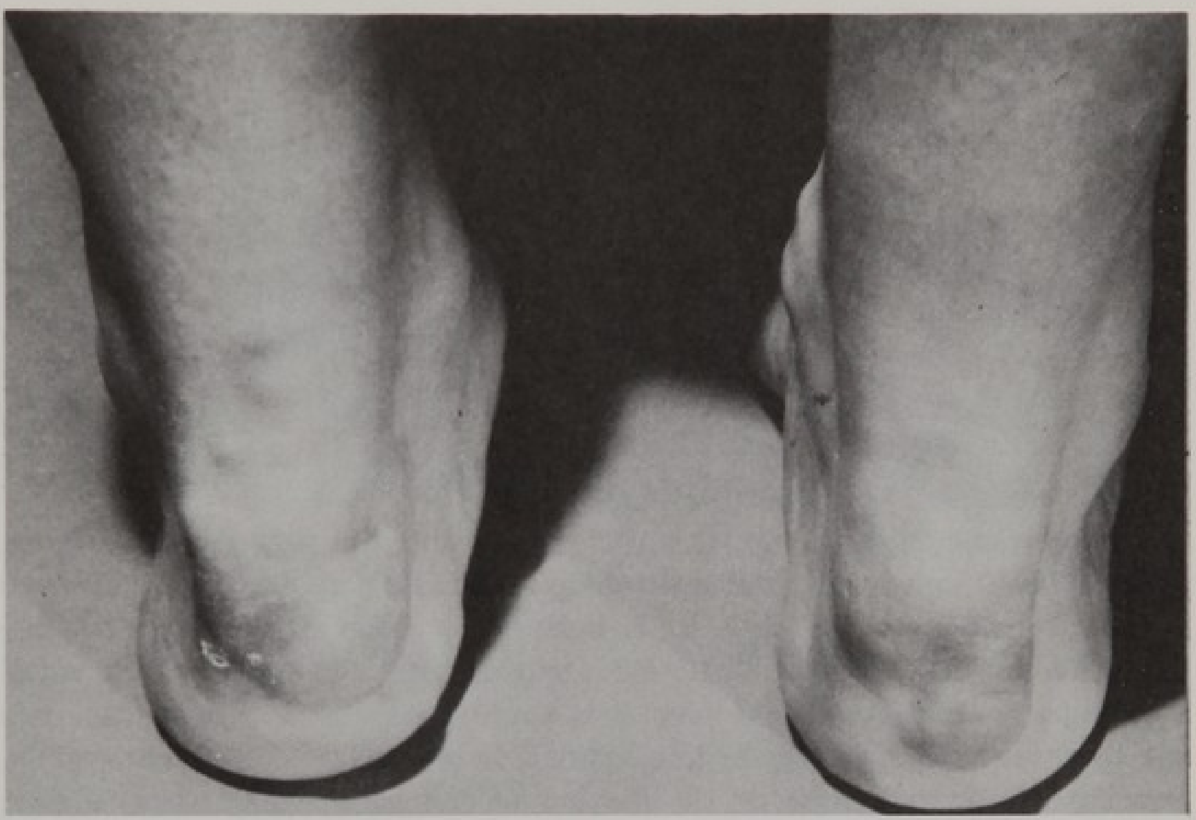

FIG. 6.–Typical far-advanced Achilles tendon xanthomas. Note the distortion of the normal anatomy with encroachment upon the calf muscles at the upper end and upon the heel at the lower end.

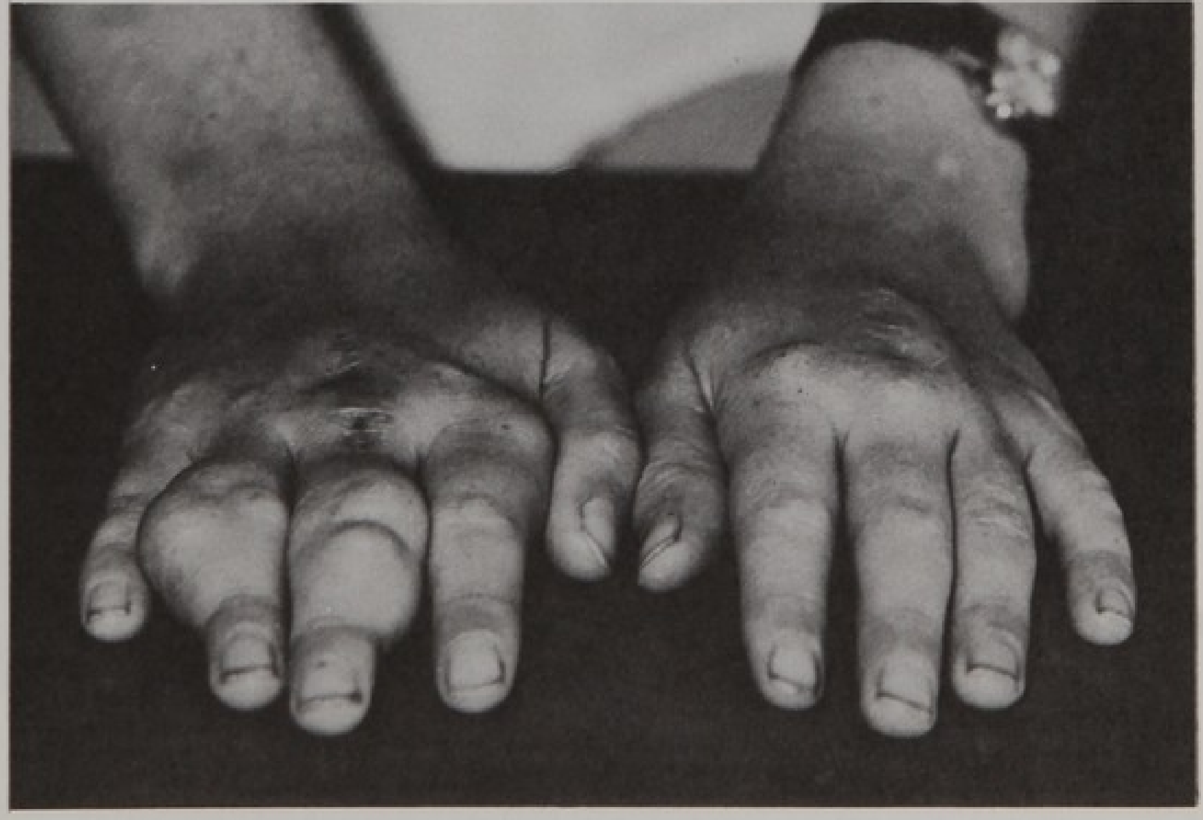

FIG. 7.–Far-advanced xanthomas of the finger extensor tendons in an adolescent.

Ocular manifestations of the disease are also quite common, arcus corneae (Fig. 8) being the most prevalent. It occurs in many patients with hyperlipidemia, not only in type II, but also in types III, IV, and V and in older, apparently normal people. Type II patients may develop arcus in the first 20 years of life, resulting finally in a dense continuous annulus around the periphery of the cornea. No matter how well developed the arcus, it never encroaches on the central portion of the cornea or affects vision. Generally, earlier development of corneal arcus correlates with greater hypercholesterolemia. Xanthelasma also occurs occasionally in patients with other types of hyperlipoproteinemia, with age, and in normal people. Like arcus, it is very common in type II patients (Fig. 9) and, generally, the younger the patient, the more severe is his hypercholesterolemia.

In severely affected patients, most often presumptive type II homozygotes, tuberous xanthomas of the elbows and gluteal folds may develop. These large lesions are subcutaneous (Fig. 10), but the skin is often affected by secondary inflammation and is discolored and atrophic over the lesions. The name, suggesting resemblance to the potato, is appropriate, for the xanthomas often are firm, resilient, and more or less bulbous. In severely affected homozygotes, peculiar raised xanthomas of the dorsum of the hand, which may be early tuberous lesions, are frequent.

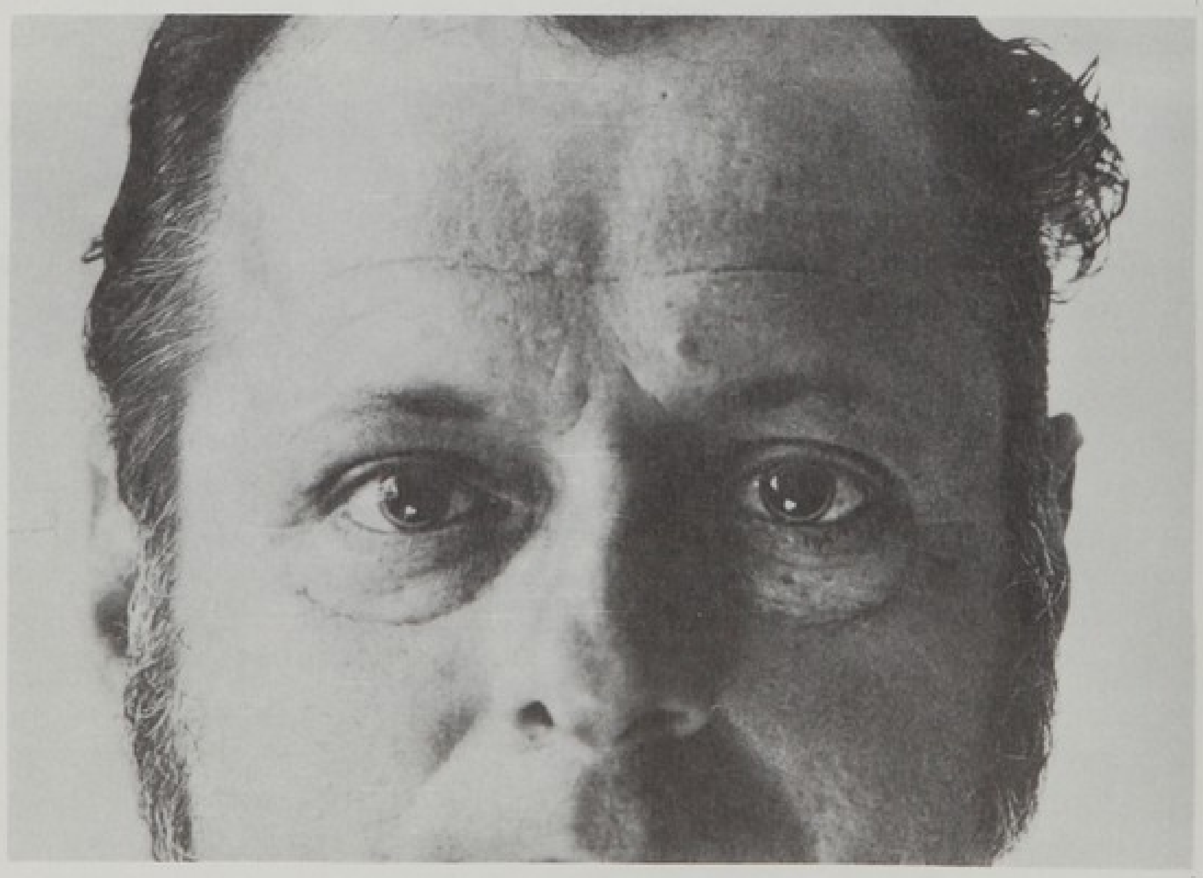

FIG. 8.–Well-developed arcus corneae. There is usually a clear zone, easily visible here, between the dense, grayish accumulation of cholesterol crystals and the edge of the cornea.

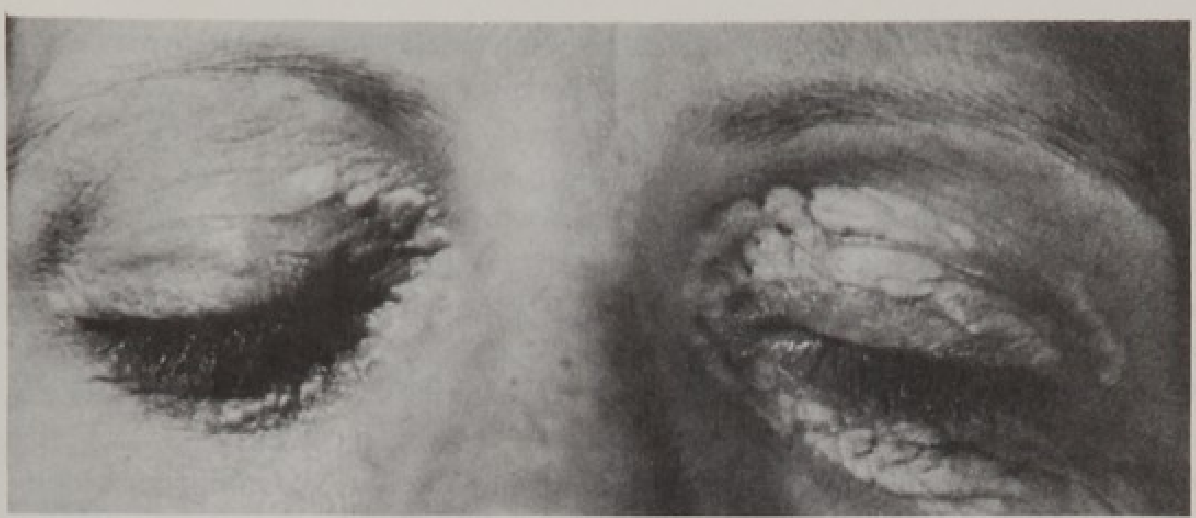

FIG. 9.–Xanthelasmas. These flat lesions are usually bright-orange in color and have a velvety feel to the touch. They are soft and quite flexible.

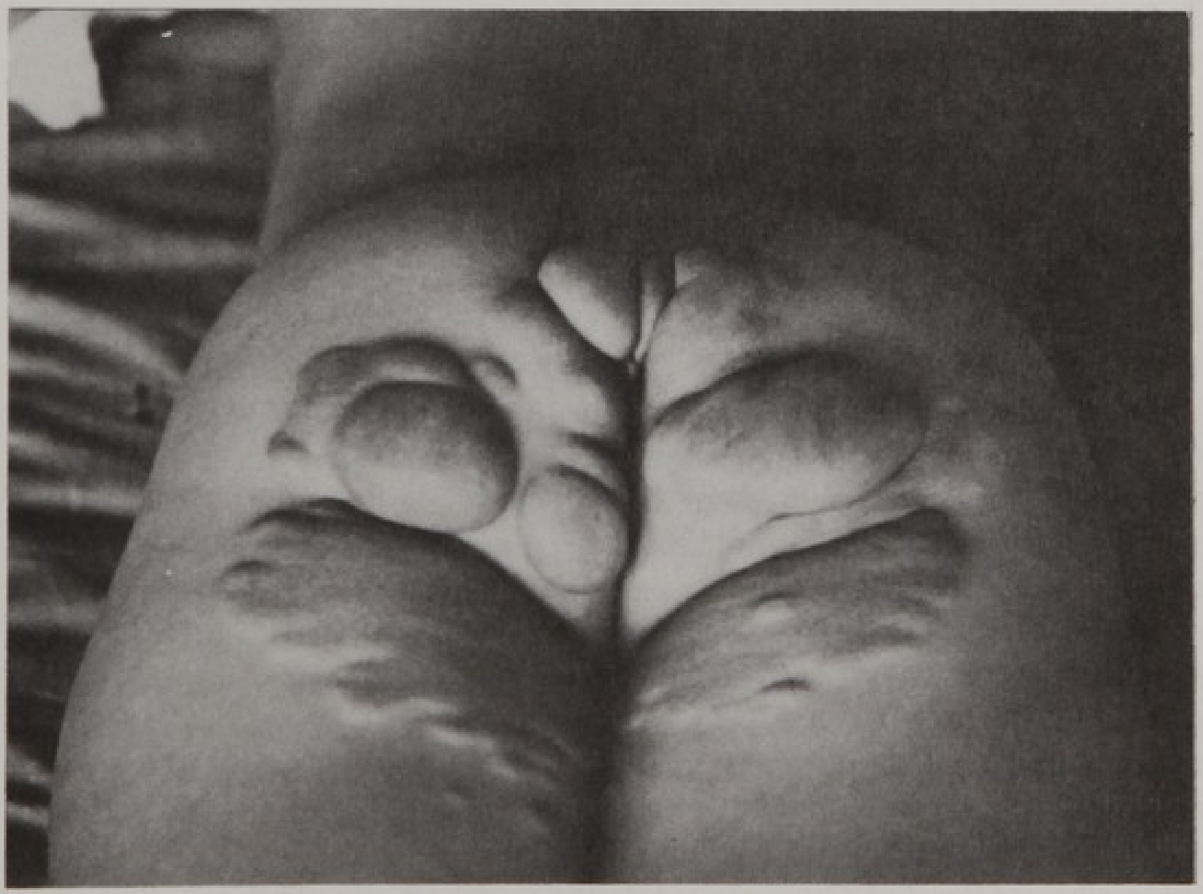

FIG. 10.–Tuberous xanthomas of the buttocks in an adolescent boy (same patient as in Fig. 7). These lesions, often seen in subjects homozygous for familial type II hyperlipoproteinemia, are also found on the elbows. They are mealy to firm in consistency, not hard, and may become the site of chronic infection from superficial skin breakdown over them.

Atherosclerosis occurs in type II early and severely. Fatal episodes may occur within the first 10 years of life. Most patients with homozygous disease are severely affected before the thirtieth year. Heterozygotes often develop coronary disease before the end of the third and usually before the end of the fourth decade. Atherosclerosis may affect any of the arterial vessels but tends to affect the coronary arteries. Most type II patients die from atherosclerotic complications. One might expect that the atherosclerosis is not that usually seen in normolipidemic patients, but rather xanthomatosis restricted to the intima. With the rare exception of prominent intimal xanthomatous lesions in severely affected homozygous patients (Stanley et al., 1965), most patients, including ours, have atheromas morphologically similar to those in normolipemic elderly subjects. A family with type II and xantomatosis has been reported (Harlan et al., 1966) in which atherosclerosis was not prominent. This is most unusual for type II patients.

Khachadurian (1968) described migratory polyarthritis in a group of Lebanese type II patients, primarily homozygotes. Of 100 type II patients reviewed by the senior author from his own clinical experience, 5 had migratory polyarthritis, although only 2 of those 5 were presumptive homozygotes. The arthritis was characterized by swelling, erythema, and pain in a large joint, usually the elbow, knee, or ankle: The arthritis was not usually accompanied by fever, but occasionally by slight lymphocytosis, increased erythrocyte sedimentation rate, and increased serum gamma globulin. After a few days, symptoms in the joint subsided. Achilles tendinitis (Glueck et al., 1968) has also been reported in this disease.

Since joint damage apparently is not permanent with hypercholesterolemic arthritis, recognition of its origin could spare patients the expense of unnecessary diagnostic procedures.

Laboratory diagnosis involves determinations of plasma lipid and lipoprotein concentrations and histologic examination of lesions. Plasma in fasting type II patients is ordinarily quite clear. Rarely, it is faintly turbid. Sometimes in severely affected patients, the plasma is deep orange due to higher-than-normal carotene concentration since plasma carotenes are carried on LDL. Plasma glycerides may be normal, but are often elevated in the range of 150 to 400 mg/dl. A type II patient with glycerides over 400 mg/dl is most unusual. Plasma cholesterol may vary from 250 to 1000 mg/dl or higher. The ratio of glycerides to cholesterol is usually one or less. The lipoprotein pattern on paper electrophoresis shows increased beta lipoproteins, often with slightly increased pre-beta lipoproteins, normal or slightly decreased alpha lipoproteins, and no chylomicrons (Fig. 11). Other electrophoretic methods give similar findings.

Chemically, the LDL, whose increased concentration in plasma is the diagnostic feature of type II, appear normal (Smith, 1962; Fredrickson and Lees, 1966; Lees, 1970b). Other metabolic parameters are usually normal in type II,

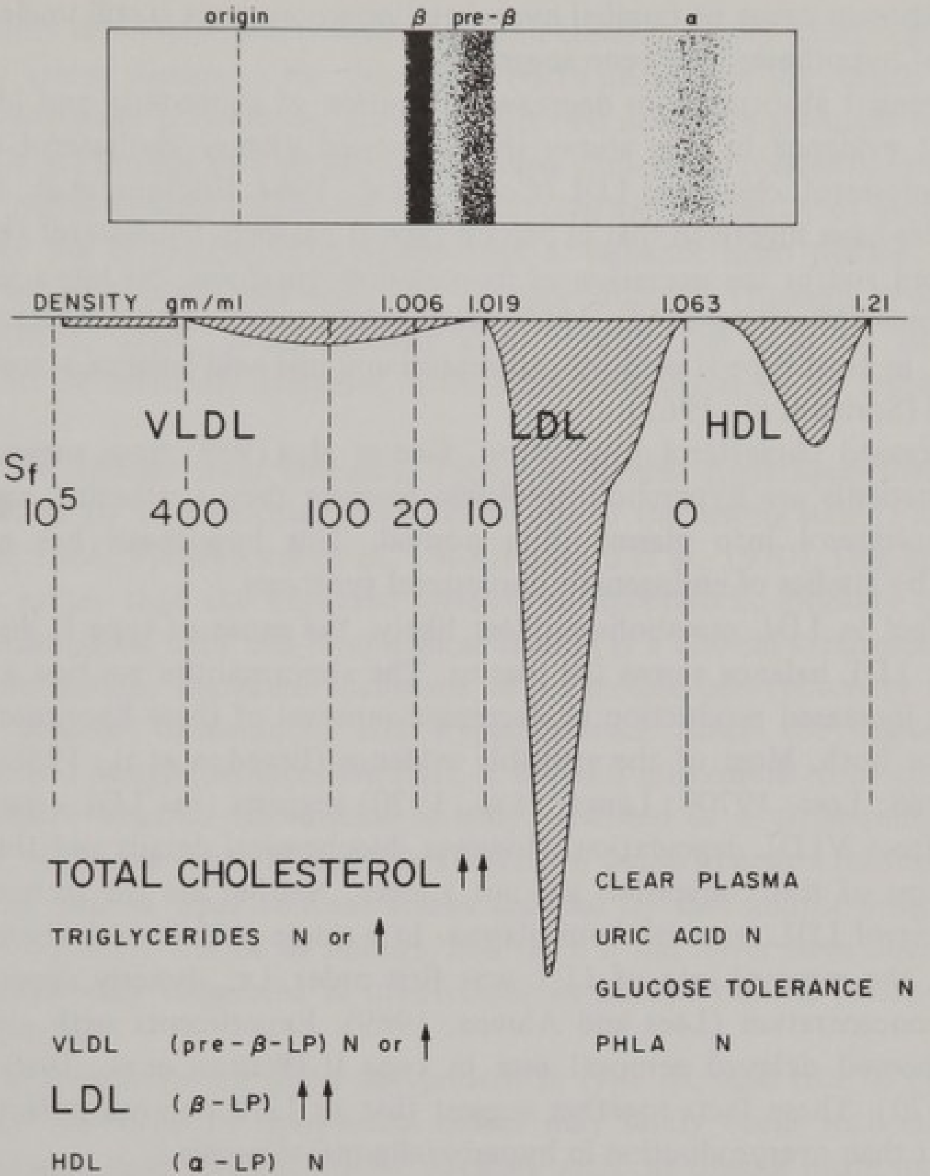

FIG. 11.–Laboratory findings in type II hyperlipoproteinemia, arranged as in Fig. 2.

including blood sugar and glucose tolerance (Glueck et al., 1969), plasma insulin, and plasma uric acid (Jensen et al., 1966). Abnormal carbohydrate inducibility (cf. type IV) is not usually found.

Tissue deposition of cholesterol is common in type II. Xanthomatosis and atheromatosis are frequent sequelae of this disease. Histologic examinations of early xanthomatous lesions reveal foam cells in the dermis. These progress to deposition of crystalline cholesterol in the skin and tendons, producing cholesterol clefts with inflammatory reaction around them (Fletcher and Gloster, 1964). Chemically, the xanthomas contain lipids of several classes, chiefly cholesterol and glycerides (Fletcher and Gloster, 1964). The arterial lesions are usually classical atheromas, although xanthomatous lesions of the aorta have been found in a severely affected case (Stanley et al., 1965).

The ultimate cause of familial hyperbetalipoproteinemia is still unclear, but a number of hypotheses have been suggested:

1. Increased absorption or decreased excretion of cholesterol and bile acids. Abundant evidence in man shows that increased dietary cholesterol increases blood cholesterol, chiefly as LDL (Connor et al., 1964; Erickson et al., 1964). A few studies have suggested that in certain type II patients, cholesterol absorption is increased and/or the excretion of its metabolic products, the bile acids, is less than normal (Miettinen et al., 1967; Wollenweber et al., 1967; Kottke, 1969). However, in most type II patients cholesterol and bile acid balance across the gut is normal (Spritz et al., 1965).

2. Increased cholesterol production. Gee et al. (1959) have suggested that type II patients are hypercholesterolemic because they synthesize and secrete more cholesterol into plasma than normal. This hypothesis has not been affirmed by studies of endogenous cholesterol synthesis.

3. Defect in LDL metabolism. Most likely, the cause of type II disease is a defect in LDL balance across the plasma. The abnormalities are best explained by either increased production or decreased removal of these lipoproteins from plasma, or both. Most of the available evidence (Bragdon et al., 1956; Carlson et al., 1968; Lees, 1970b; Langer et al., 1970) suggests that LDL arises almost entirely from VLDL degradation. However, biochemical details and the control mechanisms of this conversion are not known. Neither are the factors known which control LDL removal from plasma. In a single patient with abetalipoproteinemia, the removal rate of LDL was first order, i.e., directly dependent on plasma concentration (Lees and Ahrens, 1969). Experiments with radioactive tracers showed delayed removal rate in type II (Walton et al., 1963; Langer et al., 1970). These facts together suggest that an LDL removal defect is more important than overproduction in hyperbetalipoproteinemia.

Clearly, the definition of type II on a genetic basis is difficult (Schaefer, 1964; Thomas et al., 1964). The distribution of plasma cholesterols is skewed to the right, even in a relatively homogeneous population of similar age (Thomas et al., 1964). This is undoubtedly due to the multiplicity of environmental and genetic factors which influence plasma cholesterol, e.g., dietary cholesterol, total calories, the presence of other hyperlipidemic disorders in the population, and even stress (Grundy and Griffin, 1959). In our experience many patients with the type II syndrome have polygenic inheritance. Their hyperlipoproteinemia is characterized by increased LDL, but there is no clear Mendelian passage of the trait. Members of the same family may show all gradations of hyperbetalipoproteinemia. On the other hand, the syndromes of many patients, including almost all of those with significant xanthomatosis, seem dominantly inherited (Guravich, 1959; Hirschhorn and Wilkinson, 1959; Guravich and Venegas, 1962; Stecher and Hersh, 1949; Alvord, 1949; Leonard, 1956; Wheeler, 1957; Khachadurian, 1964; Harlan et al., 1966). The patients with a single dominant

gene usually develop hypercholesterolemia in childhood with xanthomatosis and coronary artery disease in the third and fourth decades of life; death 10 to 20 years prematurely is common. Patients with two dominant genes are sometimes born with xanthomas and usually have plasma cholesterol concentrations of 600 to 1000 mg/dl in early childhood. These patients are frequently of short stature. They may die in childhood or adolescence of ischemic heart disease. Some do survive to adulthood and the age of reproduction (Epstein et al., 1959; Khachadurian, 1964, 1968: Stanley et al., 1965). The gene for the disease appears to be relatively common, although no estimates of its frequency are available.

Type III (*Broad Beta Disease*). Type III familial hyperlipoproteinemia is characterized by an abnormal VLDL in plasma in the fasting state (Fredrickson and Lees, 1966; Fredrickson et al., 1968; Hazzard et al., 1970). This lipoprotein has beta rather than the expected pre-beta electrophoretic mobility of normal VLDL. Associated with this biochemical finding is a clinical syndrome, marked by xanthomatosis, increased incidence of atherosclerosis (coronary and peripheral), glucose intolerance, and hyperuricemia. Since the disease seems inherited as a Mendelian recessive trait, it is often impossible to be certain in a given patient whether the disease is truly inherited or is an acquired defect. However, since the type III anomaly (described below) persists throughout vast changes in plasma lipid concentrations induced by diet and/or drug therapy, since it frequently occurs in siblings, and since it has never been observed in a patient previously diagnosed as unaffected, we assume that its presence signals familial disease.

Clinical symptoms are often not present in type III, as is true of type II. In fact, since the type III lipoprotein defect may rarely occur with completely normal plasma lipid concentrations, patients with the disease might not even be detected by screening of the plasma lipids. Ordinarily, however, plasma lipid screening will detect these patients. If the patient has not been subjected to such screening, his illness is first detected when the characteristic xanthomatous deposits appear, sometimes in childhood, but usually in the third or fourth decade of life. Frequently, even the xanthomatous lesions are not noticed by the patient and his physician, nor is their true import understood. Patients may also come to a physician's attention because of clinical gout or diabetes mellitus or because of a strong family history of these or of atherosclerotic vascular disease. Finally, patients may be diagnosed when they suffer from coronary heart disease or peripheral vascular disease in the fourth and fifth decades of life.

Treatment of the lipid abnormality of type III is highly successful. Patients respond dramatically to dietary and drug therapy, frequently returning to normal plasma lipid concentrations; however, the peculiar broad beta lipoprotein usually remains detectable in the plasma. One encouraging study suggests that atheromatous disease too may regress in patients with type III, perhaps because

the histology of the obstructing lesions is different from that in the other hyperlipoproteinemias (Zelis et al., 1970).

The percentage of type III patients with xanthomas is unknown. The authors estimate that at least half of all type III patients do have them. These xanthomas include tendon, tuberoeruptive, and tuberous xanthomas, xanthelasma, and arcus corneae, and planar xanthomas of the palms. All of these except the tuberoeruptive and planar lesions are similar, at least grossly, to lesions of type II. Usually, the tuberoeruptive lesions occur only in the area around the elbow. They begin as eruptive xanthomas, small yellow, tan, or orange lesions on an erythematous base, which become chronic and enlarge to become sessile lesions 0.5 to 3 cm in size. These lesions, even when large and chronic, remain in the skin and grow outward from it, and do not involve the subcutaneous tissue. They are thus easily differentiated from the true tuberous lesion of type II disease. The planar lesions of the palms are characteristic of type III. They begin as thickening of the palmar creases which may be skin-colored or, alternatively, pigmented yellow, tan, or rarely orange. Although very subtle in their initial stages, xanthomas may grow to thick, disfiguring, and incapacitating lesions which inhibit finger movement.

Xanthomatosis in type III seems roughly proportional to the extent of hyperlipidemia. Successful treatment can effect relatively rapid regression (within months) even of advanced lesions.

As in type II, atherosclerosis is a virtually uniform finding in type III disease, occurring generally in the third through fifth decades, slightly later than in type II. Coronary heart disease and peripheral vascular disease with intermittent claudication develop with about equal frequency. Most patients die of atherosclerotic vascular disease.

Laboratory findings vary. Plasma in type III is often unremarkable in gross appearance. It may be slightly turbid during fasting, but it is only rarely milky or creamy. A thin cream collar may form during overnight refrigeration; the infranate remains turbid. Plasma cholesterol is usually elevated within the range of 300 to 600 mg/dl. Rarely, it may be as high as 1000 to 1200 mg/dl. Plasma triglycerides are usually elevated to the same levels as plasma cholesterol. The glyceride:cholesterol ratio rarely, if ever, exceeds two. The lipoprotein pattern on paper electrophoresis is not always diagnostic. Classically, there is a broad lipoprotein band extending from behind the beta-lipoprotein range forward to the pre-beta-lipoprotein area with trailing back to the origin (Fig. 12). A faint chylomicron band may be visible; the alpha-lipoprotein band appears normal. Not infrequently, however, the pattern may have an apparently normal beta-lipoprotein band merging into a normal pre-beta-lipoprotein band. In these cases the diffuseness of the bands may suggest, but not be diagnostic of, type III. A relatively simple confirmation, which should be performed whenever the diagnosis is suggested clinically, is ultracentrifugation of the plasma at its own

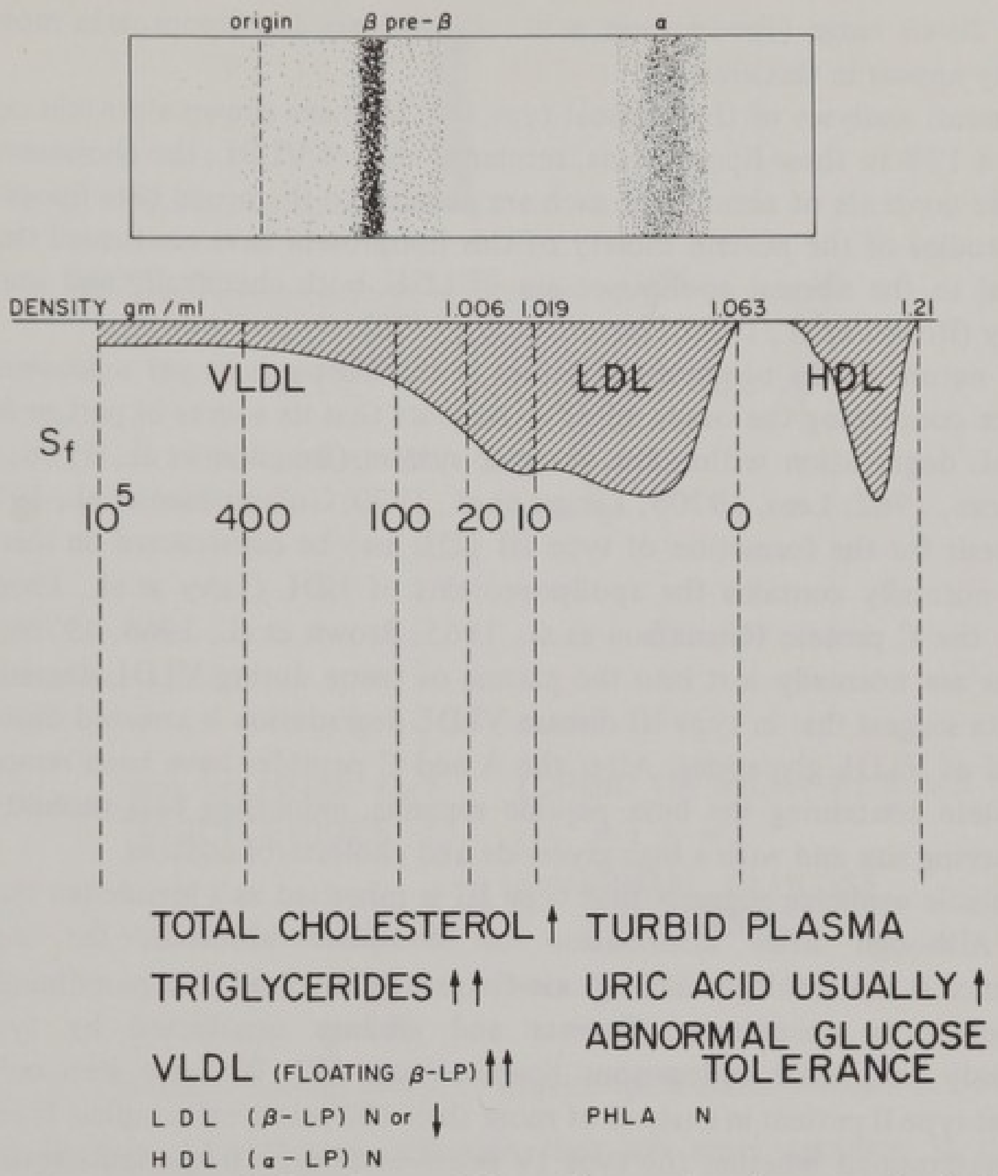

FIG. 12.–Laboratory findings in type III hyperlipoproteinemia, arranged as in Fig. 2.

density (Fredrickson et al., 1967). Normal LDL sinks at the density of plasma (1.006 g/ml), whereas normal pre-beta lipoprotein, a VLDL, floats at plasma density. When paper electrophoresis is performed with the ultracentrifugal top and bottom, the top has pre-beta mobility only and the bottom shows normal beta- and alpha-lipoprotein bands. By contrast, most of the LDL of type III patients float at plasma density and only small amounts of LDL appear in the ultracentrifugal bottom with the HDL and the other plasma proteins.

Other analytical methods give comparable findings. The broad beta-lipoprotein band is readily apparent on agarose gel electrophoresis (Noble, 1968). Perhaps the most definitive diagnosis of type III is by the analytical ultracentrifuge. The patterns produced by the lipoproteins from type III plasma do not show the normal sharp LDL peak at SF 4-8. Instead, there is a broad peak extending across the LDL and VLDL area with considerable lipoprotein in

the SF 20-40 range (Fredrickson et al., 1968); very few lipoprotein molecules normally appear in this area.

Chemical analyses of the unusual type III LDL have shown a protein content of about 12% in these lipoproteins, similar to that of VLDL; the cholesterol and glyceride contents of about 30% each are peculiar to the broad beta lipoprotein. Many studies of the protein moiety of this lipoprotein have confirmed that it is identical to the normal apolipoprotein of LDL, both chemically and immunologically (Brown et al., 1970; Hazzard et al., 1970).

The nature of the biochemical defect in the disease is as yet unknown. The evidence concerning the origin of LDL suggests that its source in part or *in toto* is VLDL degradation within the vascular system (Bragdon et al., 1956; Shore and Shore, 1962; Lees, 1970b; Langer et al., 1970; Gulbrandsen et al., 1971). A hypothesis for the formation of type III LDL may be constructed on this basis. VLDL normally contains the apolipoproteins of HDL (Levy et al., 1966b) as well as the C protein (Gustafson et al., 1965; Brown et al., 1966, 1970); these proteins are normally lost into the plasma or tissue during VLDL degradation. The data suggest that in type III disease VLDL degradation is arrested during the removal of VLDL glycerides. After the A and C peptides have been removed, a lipoprotein containing the beta peptide remains, exhibiting beta mobility, but with varying size and with a high glyceride and cholesterol content.

Available evidence suggests that type III is inherited as a Mendelian recessive trait. Although little information on complete kindreds, the authors' experience is that multiple siblings are frequently involved, but parent-and-child involvement is uncommon. Parents and siblings unaffected by type III commonly have mild endogenous lipemia (type IV). We have seen only one clear-cut type II patient in analysis of more than 100 relatives' samples. It remains to be determined whether the type IV relatives of type III patients (presumed heterozygotes for type III) can be differentiated on the basis of lipoprotein composition from other type IV patients.

Type IV (*Hyperprebetalipoproteinemia*). Perhaps the most common abnormality in our population, type IV hyperlipoproteinemia, is characterized by an increase in plasma VLDL of normal composition and mobility. LDL are normal or low in concentration and HDL are usually low (Fig. 13). Chylomicrons are usually not present, but in severe lipemia, which is rare in type IV, they may occur even in rather high concentration.

The most salient *clinical features* of type IV are its silence clinically until atherosclerotic complications appear, and its heterogeneity genetically and in laboratory presentation. Rarely, a patient with severe lipemia may have abdominal pain, eruptive xanthomas, hepatosplenomegaly, and lipemia retinalis. In most cases, however, hyperprebetalipoproteinemia is suggested by the increased plasma glycerides apparent on routine screening or in patients with clinical atherosclerotic disease. Frequently, arcus corneae and xanthelasma

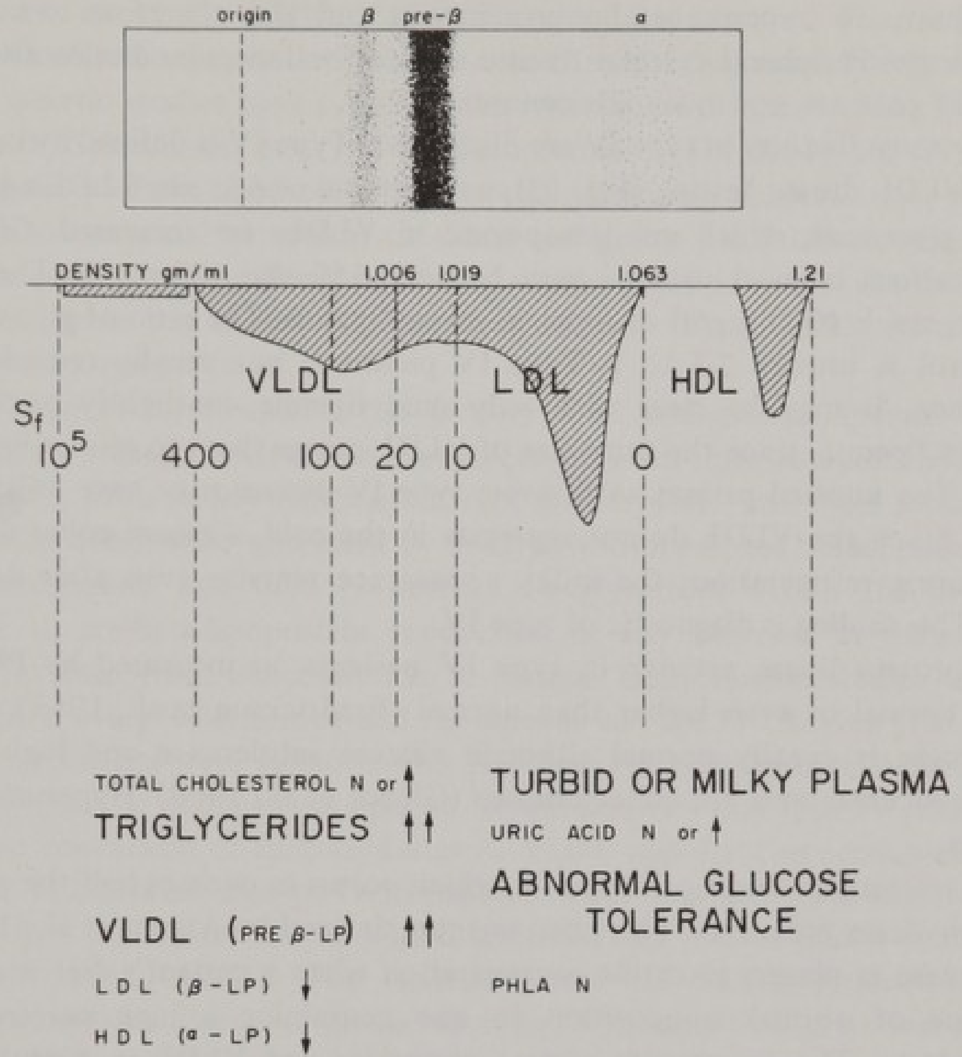

FIG. 13.–Laboratory findings in type IV hyperlipoproteinemia, arranged as in Fig. 2.

develop in type IV patients, although they are more common in type II. Ancillary features also often include obesity, abnormal glucose tolerance or overt diabetes mellitus, hyperuricemia, and hypertension.

Atherosclerosis, mainly coronary heart disease, is a frequent complication. Of the lipoprotein defects in patients with coronary disease, type IV seems most common. Many studies (Gofman et al., 1954; Besterman, 1957; Brown and Doyle, 1967; Heinle et al., 1969) have shown a striking relationship between hyperprebetalipoproteinemia and ischemic heart disease. Probably not all of this disease is familial, at least in the Mendelian sense, since many environmental factors can increase plasma VLDL. These include obesity (Albrink et al., 1962, 1965; Waxler and Craig, 1964; Ford et al., 1968), carbohydrate intake (Watkin et al., 1950; Hatch et al., 1955; Nichols et al., 1957; Antonis and Bersohn, 1961; Ahrens et al., 1961; Schonfeld, 1970), and alcohol intake (Zieve, 1958; Losowsky et al., 1963; Jones et al., 1963; Isselbacher and Greenberger, 1964; Gebbie and Prior, 1967; Kudzma and Schonfeld, 1971). However, an inherited predisposition to the effects of these environmental factors probably enhances

susceptibility to hyperprebetalipoproteinemia and also therefore to coronary heart disease. Peripheral vascular disease and cerebral vascular disease also occur in type IV patients, but much less commonly.

Laboratory findings in type IV are distinctive. Type IV is defined by increased plasma VLDL during fasting (Fig. 13), with normal or decreased LDL and HDL. Plasma glycerides, which are transported in VLDL, are increased. Glyceride concentrations in most patients vary between 150 and 400 mg/dl. They may, however, reach 1000 mg/dl or more in severe lipemia. The ratio of glycerides to cholesterol is usually 2-5 to 1. Type IV plasma is not usually remarkable in appearance. It may be clear with only mild lipemia, or slightly turbid with moderate lipemia, since the small size of VLDL causes them to refract light only poorly. The unusual patient with severe type IV disease may have milky-white plasma. Since the VLDL do not aggregate in the cold, a cream collar does not form during refrigeration; the milky appearance remains even after days and weeks. This finding is diagnostic of type IV.

Lipoprotein lipase activity in type IV patients, as measured by PHLA, is usually normal or even higher than normal (Fredrickson et al., 1963). Fasting blood sugar is usually normal although glucose intolerance and high plasma insulin concentrations are quite common (Glueck et al., 1969). Hyperuricemia is common.

A characteristic finding in type IV, which occurs in perhaps half the patients, is carbohydrate induction. This phenomenon, defined by Ahrens et al. (1961), is the increase in plasma glyceride concentration when a patient's diet is changed from one of normal composition to one containing a high percentage of carbohydrate. The increase in plasma glycerides and VLDL in type IV is an exaggeration of a normal response. Nearly all subjects on a high-carbohydrate, low-fat diet, even if they do not gain weight, increase their plasma glycerides and VLDL (Nichols et al., 1957; Ahrens et al., 1957, Antonis and Bersohn, 1961; Lees and Fredrickson, 1965a, b; Schonfeld, 1970). This response occurs regardless of the type of carbohydrate (Lees, 1967b) and is temporary, even when the diet is continued. However, plasma glyceride levels may not return to normal for up to 6 months (Antonis and Bersohn, 1961). Type IV patients tend to have an exaggerated response, with plasma glyceride rises of from 500 to over 1000 mg/dl (Glueck et al., 1969) as compared to the normal responses of 100 to 400 mg/dl (Lees and Fredrickson, 1965a). Therefore, patients with ischemic heart disease and elevated lipids must not be treated empirically with low-fat, high-carbohydrate diets, since many of them have the type IV syndrome and plasma lipid and lipoprotein concentrations may rise rather than fall on such a diet.

The nature of the inheritance of type IV is by no means clear. Since endogenous lipemia, i.e., hyperprebetalipoproteinemia, is very common and is often a secondary finding in other diseases (Fredrickson et al., 1967), genetic

studies are extremely difficult to perform and to interpret. Plasma glyceride distribution in the population is bi- or even trimodal (Schaefer, 1964). Although several genetic studies have suggested that the disease is inherited as a Mendelian dominant (Schreibman et al., 1969; Braunsteiner et al., 1968), the tremendous heterogeneity of the lipoprotein defect, with plasma glycerides varying from just barely above normal to several thousand milligrams per deciliter, suggests that the disease is polygenic. Previous clinical studies (Fredrickson et al., 1967) and a recent study in our clinic of a large single family (Fleet, 1970) are consistent with that interpretation.

The type IV syndrome is the prototype of endogenous lipemia. Pre-beta lipoproteins transport out of the liver glycerides from three sources: those stored in the liver, those newly synthesized from carbohydrate, and those formed from fatty acids which were generated by lipolysis within adipose tissue, released into the bloodstream, and then reesterified to glycerides within the liver. Any increase in pre-beta-lipoprotein production or any decrease in their removal would increase their concentration in plasma. Early kinetic studies suggested that VLDL overproduction caused the lipemia in type IV (Reaven et al., 1965). These conclusions were supported by findings that carbohydrate induction was quite common in endogenous lipemia. It was assumed that type IV patients had increased conversion of carbohydrates to VLDL and that this accounted for the elevated VLDL levels in type IV hyperlipidemia. However, recent kinetic studies suggest that the defect lies in VLDL removal from plasma (Sailer et al., 1966; Quarfordt et al., 1970). The increased rate of VLDL production on a high-carbohydrate diet is no greater in type IV patients than in normal subjects. Thus VLDL particles produced at a normal rate accumulate in the plasma because of their inability to exit at a normal rate (Quarfordt et al., 1970).

Recent studies from our laboratory (Lees, 1970a, 1970b) suggest that type IV is biochemically heterogeneous and that in some patients overproduction of VLDL and increased VLDL turnover are predominant, whereas in others a defect in removal is most prominent.

Type V (Mixed Hyperlipemia). Contrary to our first impression, the type V syndrome is relatively common (Fredrickson and Lees, 1966). It represents a small percentage of patients with ischemic heart disease (Heinle et al., 1969) and is characterized by mixed hyperlipemia (Fig. 14), with accumulation of both chylomicrons and pre-beta lipoproteins in plasma during fasting. Type V, like type III, was not clearly distinguished from other lipemias before the use of paper electrophoresis as an aid in diagnosis (Fredrickson and Lees, 1965).

The *clinical features* of five patients described in the literature (Fredrickson and Lees, 1966; Nixon et al., 1969; Cross, 1969) and those of 24 patients from the authors' experience are summarized in Table 5. The typical type V patient is born into a family with a history of diabetes. His birth, infancy, and childhood are normal. Late in adolescence, or early in adulthood, often associated with

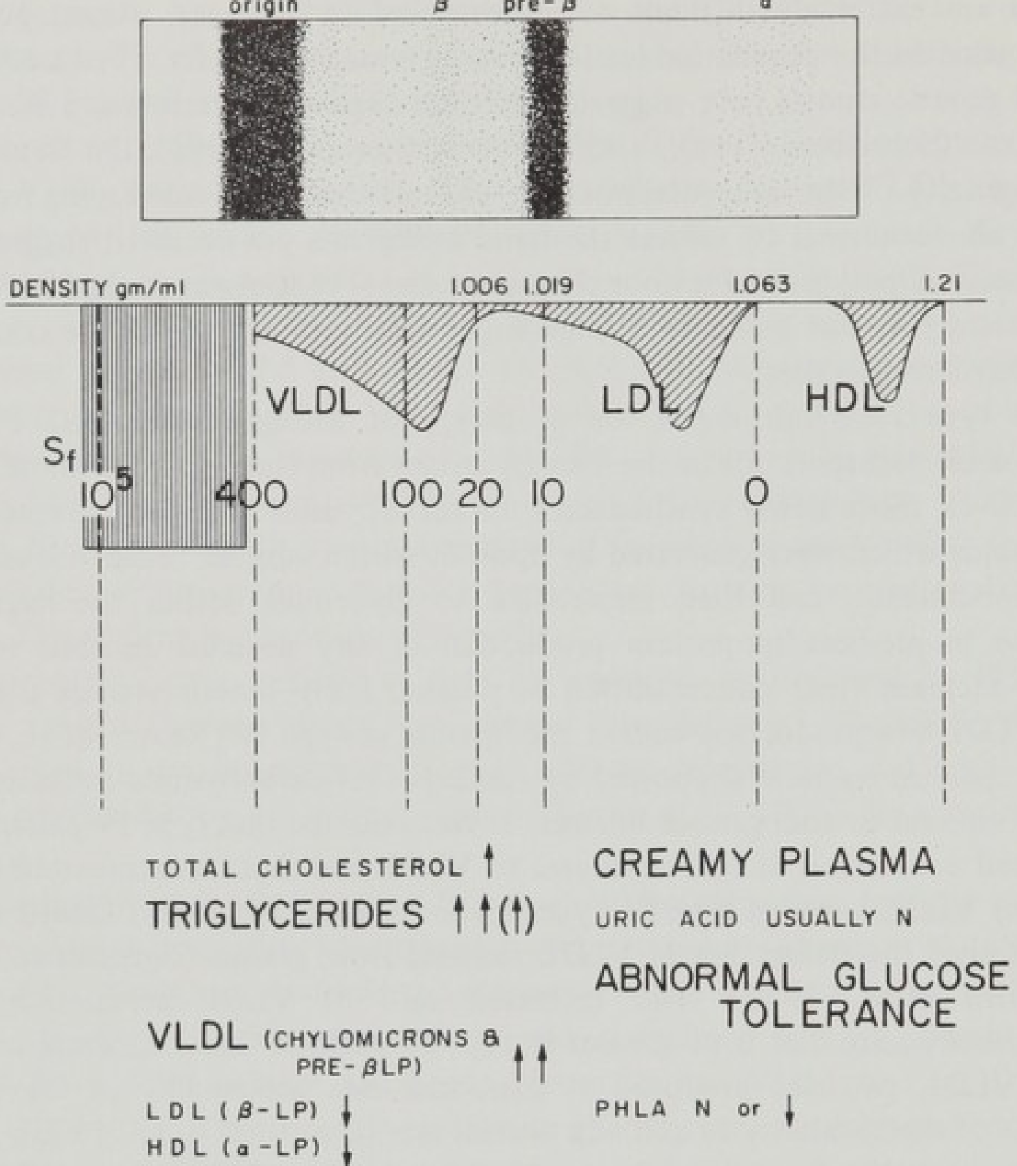

FIG. 14.–Laboratory findings in type V hyperlipoproteinemia, arranged as in Fig. 2.

weight gain, he is afflicted either with eruptive xanthomas or with episodes of abdominal pain, or with both. The abdominal pain, identical to that in type I, may mimic appendicitis, pancreatitis, or hepatic or splenic infarction. He may have one or more laparotomies. The operative findings, as in type I, reveal only slight serosal reddening, perhaps a little milky fluid in the peritoneal cavity, and usually nothing else. Physical signs are similar to those seen in type I. Enlargement of the liver is common; splenic enlargement may also occur. These organs may be tender and may remain enlarged even after successful treatment of the hyperlipidemia, although regression in size is the rule. Eruptive xanthomas similar to those of type I occur and lipemia retinalis is common. Atherosclerosis occurs at a rate somewhat greater, although probably not much greater, than normal (Slack, 1969).

Obesity is a common physical finding, and a positive family history for diabetes mellitus, gout, and ischemic heart disease are common historical

TABLE 5.–*Type V: Clinical and Laboratory Features**

References	Patient no.	Sex	Age at detection, yr	Eruptive xanthomas	Hepato- or splenomegaly	Abdominal pain	Abnormal GTT	PHLA	TC, mg/dl	TG, mg/dl	Uric acid	Atherosclerosis	Obesity
Soffer and Murray (1954)	1	M	47	–	–	+	+	Normal	372	3231		+	–
Frederickson and Lees (1966)	2	M	35	–	+	+	+	Normal	337	3059		–	+
Ibid	3	F	36	–	–	+	+	Low normal	395	1803		–	+
Ibid.	4	M	73	–	–	+	+	Low	548	3705		+	+
Nixon et al. (1969)	5	M	35	–	+	–	–	Normal	375	2533	6.5-7.1	–	+
Cross (1969)	6	M	27	+	–	+	+	Normal	408	3820		–	+
This report	7	M	14	+	+	+	–	Normal	325	2241	7.9	–	+
Ibid.	8	M	21	–	–	+		Normal	424	3340	9.2	–	+
Ibid.	9	M	40	+	–	–	+	Normal	900	4311	4.5	+	+
Ibid.	10	M	48	–	–	–	–	Normal	258	536	5.1	–	–
Ibid.	11	M	55	–	–	–	+	Normal	345	1568	5.3	+	–
Ibid.	12	F	56	+	–	–	+	Low	352	1665	5.5	+	+
Ibid.	13	F	22	+	–	+			461	1182	6.8	–	+
Ibid.	14	F	23	–	+	+	–		277	1641	5.5	–	+
Ibid	15	F	26	–	–	+	+		258	835	5.5	–	+
Ibid	16	M	26	–	–	–			348	925	7.0	–	+
Ibid.	17	M	29	–	–	–	–		525	1732	8.0	–	+
Ibid.	18	M	29	–	–	–	+		329	2922	6.3	–	+
Ibid	19	M	29	–	–	–	+		254	695	7.3	–	+
Ibid.	20	M	31	+	–	–	+		326	3036	5.8	–	+
Ibid.	21	M	31	–	–	+	+		372	1758	5.2	–	+
Ibid.	22	F	34	+	–	+	+		030	3200	5.1	–	+
Ibid.	23	M	35	+	–	–	+		270	1285		–	+
Ibid.	24	F	37	–	+	+	+		325	2132	3.8	–	–
Ibid.	25	F	38	+†	–	–	+		519	3033	5.7	–	+
Ibid.	26	M	39	+†	+	–	–		390	961	6.7	–	+
Ibid.	27	M	44	+†	–	–	–		380	700	4.9	+	+
Ibid.	28	M	45	+	–	–	–		340	2684	7.6	–	+
Ibid.	29	M	50	–	+	+	+		416	2608	4.1	+	–

* – not detected, + present; blank space indicates information is not available.

† Tuberoeruptive.

findings in type V. Frequently in adult type V patients there is considerable alcoholic intake, although the disease is quite distinct from alcoholic hyperlipemia (Zieve, 1958; Isselbacher and Greenberger, 1964; Kudzma and Schonfeld, 1971).

Laboratory findings in type V patients are various. Plasma cholesterol and glyceride concentrations in type V patients are usually elevated to the 300-to-500 and 500-to-1000-mg/dl ranges, respectively. The ratio of plasma glycerides to cholesterol is usually greater than five. The lipoprotein pattern (Fig. 14) shows a dense chylomicron band and dense pre-beta-lipoprotein band in plasma drawn during fasting. Beta and alpha lipoproteins are usually barely or not at all visible on the paper electrophoretogram. The diagnosis of type V hyperlipoproteinemia is made from the electrophoretic strip when the chylomicron and pre-beta-lipoprotein bands are of approximately equal density. Confusion may arise in laboratory diagnosis when a type IV patient has extreme hyperlipemia. In such patients, the lipoprotein electrophoresis may show a chylomicron as well as a pre-beta band. However, the pre-beta band is usually far more dense than the chylomicron band.

Plasma from a type V patient will form a dense chylomicron cream layer after refrigeration overnight and leave a milky subnate. This finding differentiates this severe hyperlipemia from the type I and IV syndromes. Other useful tests include agarose-gel electrophoresis (Noble, 1968) and PVP gradient flocculation (Gordis, 1962; Bierman et al., 1962). Rarely, the clinical and laboratory differentiation is extremely difficult and is only clarified by the patient's response to high-fat or high-carbohydrate diets (see below).

PHLA determination is often helpful in differentiating type I from type V patients if any doubt remains after clinical and laboratory evaluation. In contrast to low PHLA values in type I patients, values for patients with type V hyperlipoproteinemia usually are normal (Fredrickson et al., 1967). It has been suggested that the lipoprotein lipase present in type V plasma might be ineffective because of resistence of type V chylomicrons and pre-beta lipoproteins to lipolysis. No evidence has appeared to support such a hypothesis.

Many other laboratory values may be abnormal in type V. These include the fasting blood glucose, uric acid, and FFA. In most patients, especially older ones, glucose tolerance is abnormal (Fredrickson et al., 1967) and is associated with hyperinsulinemia (Glueck et al., 1969). Hyperuricemia occurs, especially in patients with heavy alcoholic intake. Uric acid is almost never elevated in type I patients. Some type V patients may develop acute pancreatitis, often with a hyperlipemic abdominal attack. In such cases plasma amylase and lipase and urinary amylase may be elevated.

Several type V patients in our experience have had a peculiar peripheral neuropathy. This neuropathy, which does not seem related to the state of their lipemia, presented as intermittent burning pain of the hands and would last for

hours or even days. The pain was never disabling and no perceptible changes appeared on neurological examination. Sometimes weeks or even months would pass between episodes of the pain. Although similar to diabetic neuropathy, the pain did not seem to be ameliorated in one patient by weight loss which was associated with return of glucose tolerance to normal.

Little specific information is available concerning the pathogenesis of the type V syndrome. The factors responsible for type V are probably qualitatively similar to those discussed above for type IV. However, in the type V patient, the defect in lipoprotein removal is presumably far more severe than in type IV and VLDL production is probably increased as well. Thus, both chylomicrons and VLDL accumulate in plasma. In type IV, by contrast, a partial removal defect for VLDL allows the removal of the more rapidly metabolized chylomicrons (Eaton et al., 1969) while the more slowly removed VLDL accumulate in plasma. In type I hyperlipoproteinemia, VLDL production is normal or lower than normal and chylomicron removal is completely defective. Thus, chylomicrons are the major lipoprotein accumulating in plasma.

As with types III and IV, the available genetic evidence is insufficient to determine the inheritance of type V. The syndrome appears to be more homogeneous, clinically, than does type IV. The available evidence suggests that it is inherited as a Mendelian recessive trait. Siblings of type V patients frequently are affected. Parents and children almost universally have had either normal plasma lipoproteins or the type IV pattern. The evidence suggests that patients heterozygous for the type V abnormality most likely will have a pattern of type IV hyperlipoproteinemia. It seems unlikely, from the authors' experience, that type V is simply a severe form of type IV. Rather, it seems that the type IV lipoprotein pattern may be exhibited by patients heterozygous for the type V syndrome. No family large enough to resolve the question of the inheritance of type V has yet been studied.

Secondary Hyperlipoproteinemia

Plasma lipid and lipoprotein concentrations similar to those seen in the primary familial diseases reviewed here are commonly secondary features in a variety of other diseases (Fredrickson et al., 1967; Glueck et al., 1969; Lees et al., 1970; Gallin et al., 1969). The possibility of underlying disease of another sort must always be excluded before the diagnosis of primary hyperlipoproteinemia is made. Ancillary metabolic tests are of great value both in ruling out other disease and in confirming hyperlipoproteinemia. Such tests include glucose tolerance, plasma uric acid, plasma free fatty acids, and plasma protein electrophoresis.

The most prevalent secondary lipemia is alcoholic lipemia. It is suspected when the history and physical examination yield evidence of high alcoholic

intake and is confirmed by the almost invariable elevations of plasma uric acid and free fatty acids. Perhaps the second most prevalent cause of secondary hyperlipoproteinemia is renal insufficiency (Losowsky and Kenward, 1968; Bagdade et al., 1968b). The lipemia of renal insufficiency may be exaggerated by extracorporeal dialysis.

The diagnosis of primary hyperlipoproteinemia is always more certain when it is demonstrably familial. We consider family screening for hyperlipoproteinemia routine in the medical work-up of a patient. Not only does it help confirm the individual patient's diagnosis, but also family screening often identifies previously undiagnosed, often young patients in whom early treatment may prevent some of the cardiovascular complications.

Diagnosis of the Familial Hyperlipoproteinemias

The reader is referred to other reviews for the details of and methods for diagnosis of the hyperlipoproteinemias (Fredrickson and Lees, 1966; Fredrickson et al., 1967; Hatch and Lees, 1968). We repeat here certain factors in patient sampling critical to proper diagnosis.

The patient must be on a normal American diet for a minimum of 7 to 14 days before sampling. In addition, body weight must be stable. If the patient has recently gained or lost weight, false-positive or false-negative conclusions may be reached. Unusual diets high in saturated fat and cholesterol or high in carbohydrate may likewise give false-positive values. Pregnancy is characterized by a physiologic lipemia; blood sampling in pregnant patients must be deferred until 1 to 2 months after delivery.

Subjects must be fasting when blood is drawn for lipid and lipoprotein analysis; otherwise their condition may be falsely diagnosed as hyperlipoproteinemia. Normal plasma lipid and lipoprotein concentrations are defined as those present after a 12- to 16-hour overnight fast (Fredrickson et al., 1967; Hatch and Lees, 1968). We have found that patients will readily cooperate if the purpose of the studies and the need for fasting are explained. It is important to note that plasma glyceride concentrations reach a peak 4 to 6 hours after a meal, and sampling then is maximally misleading.

For accurate assessment of plasma lipoprotein concentrations, the mean of three, or at very least two, values must be used. Plasma lipid and lipoprotein concentrations are very labile. They represent energy in transit and vary with diet, exercise, and stress. A single set of lipid and lipoprotein values may be very misleading. If two disparate values are obtained, a third value must be obtained to determine the most consistent plasma lipid and lipoprotein concentrations.

Treatment of Hyperlipoproteinemia

Many diets and drugs are now available for the treatment of hyperlipoproteinemia. In nearly all patients, plasma lipid and lipoprotein levels can be

brought to normal or nearly to normal. Further research in the hyperlipoproteinemias must determine whether such treatment reduces the rate of atherogenesis. Those who deal with these diseases clinically may refer to a recent review for the details of treatment (Lees and Wilson, 1971).

CONCLUSIONS

The clinical features of the hyperlipoproteinemias have been studied in detail over the past few years. As is evident above, little is yet known about the genetic factors which determine plasma lipid and lipoprotein concentrations in these diseases, or even in the general population. We hope that the next 10 years will see the application of the clinical and laboratory methods developed within the last 10 years to the study of these determinants. We repeat in closing that the importance of such understanding transcends the study of lipid and lipoprotein metabolism. The best single predictor of coronary disease risk, the major killer of our population, is the determination of plasma cholesterol concentration (Kannel et al., 1971).

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CHAPTER 7

Advances in Pharmacogenetics

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Many new developments in pharmacogenetics have occurred since this subject was last reviewed in this series (Motulsky, 1964). In the past, the term pharmacogenetics was used to refer to clinically significant consequences of hereditary variations in the handling of drugs (Vesell, 1969), and pharmacogenetic studies in man centered almost exclusively on a few inborn errors of metabolism involving enzymes concerned with drug biotransformation. The scope of pharmacogenetics has now enlarged considerably as a result of recent approaches to the therapy of certain hereditary disorders and to the genetic control of large individual differences in rates of drug metabolism.

Rapid expansion of pharmacogenetics during the past few years necessitates restriction of this review to conditions affecting man, although books on pharmacogenetics have appeared containing extensive material based on studies in experimental animals (Kalow, 1962; Meier, 1963). Because of many investigations in this area, pharmacogenetic studies in experimental animals require a separate review. One reason for the development of experimental pharmacogenetics is recognition that pharmacological effects often vary markedly with the strain or species (Quinn et al., 1958), and even in a given strain or species as a result of only a single amino acid substitution in a protein controlling drug disposition. Therefore, different responses to a therapeutic agent by various species, strains, or enzyme preparations can serve as effective probes to explore basic mechanisms of drug action. Because of these pharmacogenetic differences, it is advisable to perform metabolic, toxicologic, and carcinogenic studies of new drugs in at least two species. Frequently encountered strain or species differences in drug disposition and toxicity diminish the hope of finding an animal model resembling man in these respects, while they correspondingly increase the problem of adequately testing drugs for deleterious effects in animals prior to human use. Several recent symposia and volumes illustrate the current interest in this new field and the large number of new approaches being taken in pharmacogenetics (LaDu, 1965; LaDu and Kalow, 1968; Vesell, 1971; Vesell, 1972).

HISTORY

In 1957. Motulsky emphasized that certain forms of drug toxicity were produced by hereditary disorders affecting drug-metabolizing enzymes. He described several examples of such genetically controlled abnormal reactions to drugs and distinguished them from toxic reactions caused by immunological mechanisms, some of which, he pointed out, might eventually be shown to

exhibit a hereditary component. Furthermore, Motulsky recognized that the existence of this group of genetic aberrations, clinically asymptomatic until drugs were administered, provided pertinent models for demonstrating the interaction of heredity and environment in the pathogenesis of disease and that these hereditary drug toxicities might be related to susceptibility or resistance to other diseases. Vogel coined the term pharmacogenetics in 1959, and Kalow wrote a stimulating book on the subject in 1962. These works served to focus interest in several hereditary conditions then recently discovered through unusual responses elicited only by the administration of various drugs.

Pharmacogenetic disorders are not an entirely new branch of pharmacology or genetics. They should be considered a special type of inborn error of metabolism involving those proteins that function in drug metabolism or drug action. Within this context the origins of pharmacogenetics may be traced back to the first decade of this century when Sir Archibald Garrod recognized that man's chemical individuality extended well beyond the few inborn errors of metabolism that he had investigated to a host of reactions to foods and drugs. However, the underlying principle of individual variations in drug response upon which pharmacogenetics is based must have been perceived earlier; astute physicians probably recognized since the writing of the first historically documented prescription more than 4000 years ago that a drug administered in the same dose and by the same route can produce markedly different therapeutic and toxic effects in various individuals. Paul D. White (1971) maintains that in his 60 years of practice the great majority of his patients exhibited some unusual, but not pathological, response on either side of an expected mean; and he states that this observation applies to individual reactions to foods and drugs, thereby illustrating the old adage that one man's meat is another man's poison. Pharmacogenetics attempts to assess and define the nature of the hereditary component of clinically significant individual variations in the disposition and effects of drugs and also is concerned with the therapy of genetic disease. Genetic factors should be carefully distinguished from the multiple environmental contributions to such variations, including certain cardiovascular, hepatic, and renal disorders, as well as exposure to agents that enhance or inhibit drug metabolism.

DEFINITIONS

In 1957 when the conceptual foundations of pharmacogenetics were being laid, only a few examples of pharmacogenetic conditions were available. These included acatalasia, suxamethonium sensitivity, slow inactivation of isoniazid, inability to taste phenylthiourea, and a self-limited hemolytic anemia occurring after ingestion of various drugs, later discovered to be caused by glucose-6-phosphate dehydrogenase (G-6-PD) deficiency. It was anticipated in 1957 that the increasing exposure of large populations to various therapeutic agents and the

development of highly sensitive techniques for measuring small amounts of drugs in biological fluids would eventually lead to the discovery of many new pharmacogenetic disorders. With this expectation the term pharmacogenetics was initially employed rather narrowly to refer to hereditary disorders revealed solely by the use of drugs. This definition excludes from consideration several hereditary diseases that were well described before discovery of their exacerbation by certain drugs: diabetes mellitus by adrenocortical steroids; gout by thiazide diuretics; and porphyria by barbiturates or alcohol. In the past 16 years only rare and isolated examples of new pharmacogenetic conditions have been reported; in some cases the mode of inheritance of a putative new pharmacogenetic disorder could not be firmly established from the few small pedigrees available, as in the examples of bishydroxycoumarin sensitivity (Solomon, 1968), diphenylhydantoin toxicity (Kutt et al., 1964a), and phenacetin intolerance (Sahidi, 1968) (see Table 1). However, by way of contrast, two extensive pedigrees of one new pharmacogenetic entity, warfarin resistance, have been published (O'Reilly et al., 1964; O'Reilly et al., 1970).

Clearly, adverse reactions to drugs are common; few new therapeutic agents lack side effects, on either the skin, gastrointestinal tract, blood, central nervous system, cardiovascular system, endocrine organs, or genitourinary tract. However, the hereditary nature of these untoward responses remains to be established. Perhaps in the future some cases of blood dyscrasias after chloramphenicol, antibody formation to penicillin and other drugs, extrapyramidal symptoms after prochlorperazine, a syndrome resembling disseminated lupus erythematosus appearing after hydralazine administration, or the rare thrombophlebitis in women receiving progestational antifertility agents will be shown to be familial and their mode of inheritance elucidated.

Because so few new pharmacogenetic conditions have been discovered in the past 16 years, a more flexible definition of pharmacogenetics than the initial proposal discussed above seems indicated. On the other hand, too general and all-embracing a definition may not prove sufficiently discriminating to be useful. The view that pharmacogenetics deals with drug responses and their modification by hereditary influences was offered by Motulsky in 1964 and is appealing. However, it fails to exclude many pharmacological phenomena since enzymes are frequently involved in each process affecting the disposition of every drug, including drug absorption, distribution, biotransformation, and excretion, and since genetically controlled individual variations probably occur at each of these levels. To limit pharmacogenetics to clinically significant consequences of hereditary variations in drug metabolism or response may appear to be more of a restriction on the definition of Motulsky (1964) than it is in practice, since genetically controlled variations in the absorption, distribution, biotransformation, or excretion of any drug impose potential clinical consequences on its therapeutic and toxic effects. Broad as these definitions are, they require amend-

ment to include the important new area of pharmacogenetics that deals with gene therapy or the provision of new, specific genetic information to permit mutant cells to synthesize normal, instead of defective, enzyme. Some novel approaches to this pharmacogenetic problem will be described at the end of this review and have appeared in several recent symposia on pharmacogenetics (Osterman et al., 1971; Aposhian, 1972).

In summary, pharmacogenetics offers a fresh approach to the study of pharmacology and therapeutics by subjecting the classical phenomena within these disciplines to formal genetic analysis.

DEVELOPMENT OF INCREASINGLY POTENT DRUGS

Early records indicate that many thousand years ago man consumed for therapeutic purposes naturally occurring compounds, which fortunately were generally of low potency; only in the past 20 years have exceedingly potent drugs been developed. In this short period of two decades large populations have received many new potent drugs, and the trend toward the development of increasingly potent drugs continues. Eventually, such progress might result in making a small dose of most drugs therapeutically efficacious without altering the activity of the hepatic microsomal enzyme system that metabolizes them. This accomplishment would have as a beneficial effect elimination of the principal problems in pharmacogenetics and in the field of drug interactions. However, this dream, shared for different reasons by Ehrlich and the homeopaths, remains unrealized. At the present time, in order to achieve therapeutic effects, the physician must administer drugs in such high doses that biotransformation by drug-metabolizing enzymes not only occurs but is desirable to avoid adverse drug reactions. Administered chronically, these doses may induce the enzymes required to detoxify certain drugs, thereby lessening their therapeutic effectiveness through reduction in their duration of action, which in turn necessitates an increase in their dose. Certain agents inhibit the metabolism of other compounds, producing toxicity by drug accumulation.

VARIATIONS IN DRUG RESPONSE

One important adverse effect of the administration of many new potent drugs to large populations is the selection from the exposed population of individuals with a mutant, catalytically less efficient form of the enzyme required to metabolize the drug. After such individuals take the "normal" dose of the drug, their aberrant enzyme is often evident by the appearance of some type of drug toxicity. Pharmacogenetics emerged as an attempt to understand and deal with this situation. The field of pharmacogenetics has grown significantly since the mid-1950s when less than a dozen entities classifiable under this heading were identi-

TABLE 1.–*Twelve Pharmacogenetic Conditions with Putative Aberrant Enzyme, Mode of Inheritance, and Drugs That Can Elicit the Signs and Symptoms of the Disorder*

Name of condition	Aberrant enzyme and location	Mode of inheritance	Drugs that produce the abnormal response
Genetic conditions probably transmitted as single factors altering the way the body acts on drugs			
1. Actalasia	Catalase in erythrocytes	Autosomal recessive	Hydrogen peroxide
2. Slow inactivation of isoniazid	Isoniazid acetylase in liver	Autosomal recessive	Isoniazid, sulfamethazine, sulfamaprine phenelzine, dapsone, hydralazine
3. Suxamethonium sensitivity or atypical pseudocholinesterase	Pseudocholinesterase in plasma	Autosomal recessive	Suxamethonium or succinylcholine
4. Diphenylhydantoin toxicity due to deficient parahydroxylation	? Mixed function oxidase in liver microsomes that parahydroxylates diphenylhydantoin	Autosomal or X-linked dominant	Diphenylhydantoin
5. Bishydroxycoumarin sensitivity	? mixed function oxidase in liver microsomes that hydroxylates bishydroxycoumarin	Unknown	Bishydroxycoumarin
6. Acetophenetidin-induced methemoglobinemia	? mixed function oxidase in liver microsomes that deethylates acetophenetidin	Autosomal recessive	Acetophenetidin
Genetic conditions probably transmitted as single factors altering the way drugs act on the body			
1. Warfarin resistance	? altered receptor or enzyme in liver with increased affinity for vitamin K	Autosomal dominant	Warfarin

2. Glucose-6-phosphate dehydrogenase deficiency, favism, or drug-induced hemolytic anemia	Glucose-6-phosphate dehydrogenase	X-linked incomplete dominant	A variety of analgesics [acetanilide, acetylsalicylic acid, acetophenetidin (phenacetin), antipyrine, aminopyrine (Pyramidon)], sulfonamides and sulfones [sulfanilamide, sulfapyridine, N_2-acetylsulfanilamide, sulfacetamide sulfisoxazole (Gantrisin), thiazolsulfone, salicylazosulfapyridine (Azulfadine), sulfoxone, sulfamethoxypyridazine (Kynex)], antimalarials [primaquine, pamaquine, pentaquine, quinacrine (Atabrine)], non-sulfonamide antibacterial agents [furazolidone, nitrofurantoin (Furadantin), chloramphenicol, *p*-aminosalicylic acid], and miscellaneous drugs [naphthalene, vitamin K, probenecid, trinitrotoluene, methylene blue, dimercaprol (BAL), phenylhydrazine, quinine, quinidine]
3. Drug-sensitive hemoglobins: (a) Hemoglobin Zurich	Arginine substitution for histidine at the 63 position of the β chain of hemoglobin	Autosomal dominant	Sulfonamides
(b) Hemoglobin H	Hemoglobin composed of four β chains	Autosomal recessive	Same drugs as listed for G-6-PD deficiency
4. Inability to taste phenylthiourea or phenylthiocarbamide	Unknown	Autosomal recessive	Drugs containing the N–C=S group such as phenylthiourea, methyl and propylthiouracil
5. Glaucoma due to abnormal response of intraocular pressure to steroids	Unknown	Autosomal recessive	Corticosteroids
6. Malignant hyperthermia with muscular rigidity	Unknown	Autosomal dominant	Such anesthetics as halothane, succinylcholine, methoxyfluorane, ether, and cyclopropane

fied. Progress has consisted less in the description of entirely new conditions than in the recognition of variant forms of the old entities and in further biochemical, genetic, and clinical characterization of these disorders. A new area in pharmacogenetics that has stimulated much current activity was opened by the recognition that large differences among healthy, nonmedicated individuals in their rates of metabolism of commonly used drugs are primarily under genetic control and are negligibly affected by environmental factors (Vesell and Page, 1968a, 1968b, 1968c).

Each of the pharmacogenetic conditions shown in Table 1 behaves as a single factor probably resulting from a single-point mutation transmitted in classical Mendelian fashion. At some of these loci multiple mutant alleles have been detected. These pharmacogenetic entities are in fact inborn errors of metabolism affecting drug-metabolizing enzymes. Several are extremely rare, like most inborn errors of metabolism; bishydroxycoumarin sensitivity, deficient parahydroxylation of diphenylhydantoin, and warfarin resistance have been reported thus far in only one or two families. However, more extensive population screening could reveal that these pharmacogenetic conditions are much more common than currently believed. In any event, other conditions listed in Table 1, such as G-6-PD deficiency and slow inactivation of isoniazid, occur commonly; and in certain populations the gene frequency of the "aberrant" allele is higher than that of the "normal" allele (Vesell, 1969). With the exception of G-6-PD deficiency, problems of drug toxicity arise from these pharmacogenetic conditions when the individual with the mutant enzyme receives only a single type of drug. The most notable exception, G-6-PD deficiency, produces hemolysis after administration of many commonly used drugs, although in the mutant form encountered most frequently in the United States, the resultant hemolysis is mild and self-limited, even in the face of continued drug administration. The point is that conditions listed in Table 1 do not constitute a major threat of drug toxicity to many individuals. However, since the early 1950s, it has been recognized that large differences exist among individuals in rates of plasma decay of commonly used drugs. For example, the decay from plasma of such commonly used drugs as phenylbutazone (Burns et al., 1953), antipyrine (Brodie and Axelrod, 1950), and bishydroxycoumarin (Weiner et al., 1950) exhibit 3- to 10-fold variations among individuals. These differences among individuals, generally observed after administration of a single oral dose of the drug, are larger than the ranges observed for many routine laboratory studies; and they do constitute a major therapeutic danger to the population receiving drugs according to the currently employed method based almost exclusively on body weight. For example, the patient who clears a drug rapidly from the body would require more of the drug to achieve a therapeutic effect than the patient of intermediate clearing capacity. However, toxicity could result from administration of the usual drug dosage to a patient with a very low ability to remove the drug. In this

individual with low clearing capacity, drug accumulation resulting from chronic drug administration could produce toxicity. To avoid these extremes of drug toxicity and failure to derive therapeutic benefit, in the slow and rapid drug clearer, respectively, abandonment of drug administration solely according to body weight and individualization of drug doses based on measurements of drug concentrations in blood are advocated (Vesell and Passananti, 1971). By no currently available method other than measurement of drug concentrations in blood can the physician distinguish among his patients with respect to their drug-clearing capacities and administer drugs according to their individual needs.

Investigation of the responsiveness of the general population to a drug in terms of the amount of a drug required to produce a given effect may take the form of a continuous unimodal distribution curve or of a discontinuous polymodal curve (Fig. 1). Until recently, studies of drug responses that yield a normal or continuous distribution curve have been almost entirely ignored in pharmacogenetic investigations. To construct unimodal, Gaussian distribution curves large populations are required, and genotypes are difficult to derive from such curves, although theoretically for polygenically controlled traits children should exhibit values midway between those of their parents. In contrast, discontinuous curves of response obtained from disorders transmitted as Mendelian dominants or recessives are more easily analyzed because each discrete curve generally corresponds to a different genotype. In other words, the mutant genes and their corresponding phenotypes segregate both in pedigrees and in the distribution curves. Figure 1, adapted from Kalow (1962), compares the normal distribution

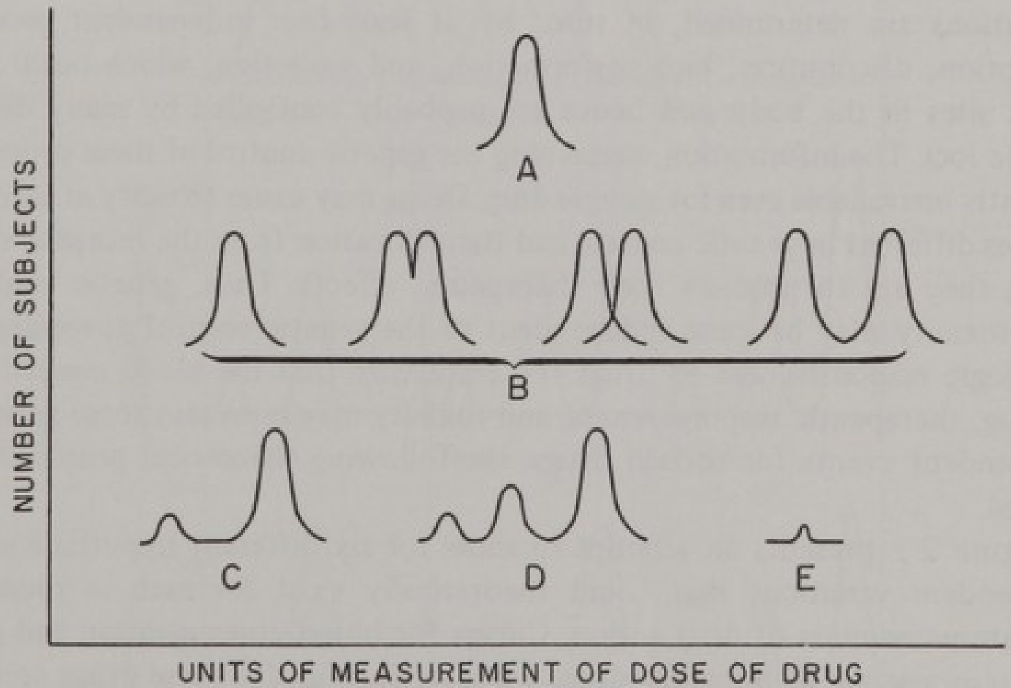


FIG. 1.–Types of frequency distribution (A-E) for the responsiveness of individuals to various drugs, as modified from Kalow (1962).

curve obtained for graded metrical characters, typically under polygenic control (curve A), with the discontinuous multimodal curves for traits transmitted as Mendelian recessives and dominants. Curve E, though unimodal and continuous, actually indicates a response by individuals possessing a single-point mutation for a genetically transmitted polymorphic trait, a response which normal individuals do not exhibit. Such examples include hemolysis in some individuals with glucose-6-phosphate dehydrogenase deficiency after administration of antimalarials, or acute arthritic attacks in certain individuals with the gene for gout after receiving thiazide diuretics, or abnormal blood glucose tolerance curves in individuals with diabetes mellitus after receiving steroids. Figure 1 includes under group B a unimodal curve, although all other examples under category B are multimodal. Because it may actually conceal genetic heterogeneity, this unimodal curve is included in group B. After more sensitive biochemical tests are performed, this unimodal curve may be shown to be composed of several genetically and phenotypically distinguishable components. For example, the broad overlapping curve obtained on plotting the serum pseudocholinesterase activity of a large population is separable into three discrete curves with the use of the enzyme inhibitor dibucaine.

The situation depicted in Fig. 1 is oversimplified because its suggests that drug responsiveness is a single trait or factor determined by alleles at one genetic locus or even, in the case of polygenic inheritance, by alleles at several separate loci. Although this may be true in some cases, the situation in most cases is more complex because the physiologic responses produced by many drugs are determined by the concentration of that drug at cellular receptor sites. These concentrations are determined, in turn, by at least four independent processes: absorption, distribution, biotransformation, and excretion, which occur at different sites in the body and hence are probably controlled by many different genetic loci. The information concerning the genetic control of these processes is currently unavailable even for a single drug. Drugs may cause toxicity at intracellular sites different in genetic control and tissue location from the receptor sites on which they act to produce their therapeutic effects. Thus, genetic control of drug toxicity may be quite independent of the genetic control governing pharmacologic responsiveness to drugs. To emphasize that the blood concentration of drug, therapeutic responsiveness, and toxicity may represent three genetically independent events for certain drugs, the following theoretical possibilities are offered.

Figure 2 represents an attempt to show for six different hypothetical drugs independent variations that could theoretically exist for each of these three separate parameters of drug action. Curves for blood concentration and physiologic response are identical for drugs 1 and 4; thus far, for these drugs prediction of the extent of the body's therapeutic reaction could be made simply from a knowledge of drug concentrations in blood. Although this situation obtains for

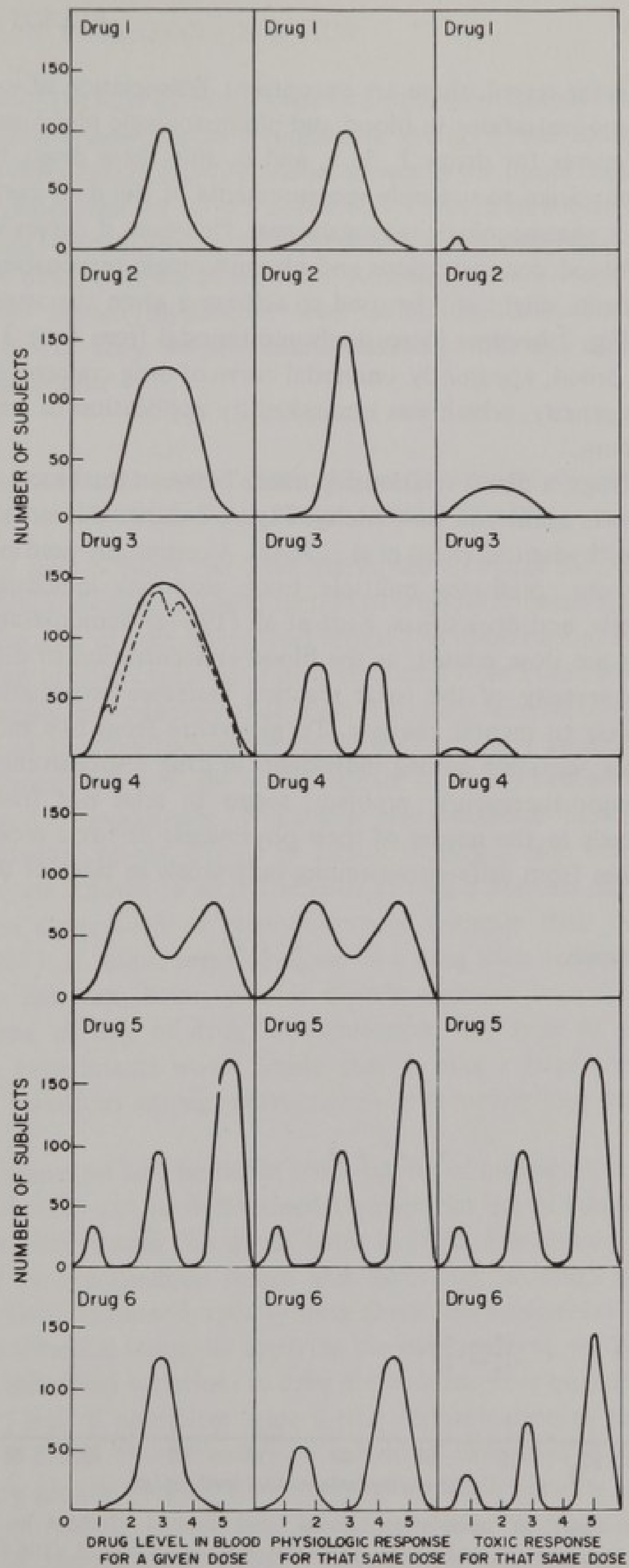


FIG. 2.–Relationship, for six different hypothetical drugs, between drug concentration, physiologic response, and toxic response, all for the same dose of drug tested in a large number of subjects.

most drugs thus far tested, there are exceptions. Dissociation of varying extents between drug concentrations in blood and pharmacologic responsiveness is illustrated by the curves for drugs 2, 3, 5, and 6. For these drugs it might seem unwise for a physician to use only measurements of the drug concentration in blood to predict pharmacologic responsiveness. However, if curves were available to show how blood concentrations and pharmacologic responsiveness differed, such measurements might still be used to achieve a given therapeutic response. The curves in Fig. 2 become increasingly multimodal from drug 3 to drug 5. In drug 3, a very broad, apparently unimodal curve of drug concentration in blood conceals heterogeneity, which was unmasked by application of a more sophisticated assay system.

For many drugs, a direct relationship exists between the blood concentration of the therapeutic agent and its toxicity. Figure 3 shows this situation to be the case for diphenylhydantoin (Kutt et al., 1964a). A commonly used anticonvulsant, diphenylhydantoin, produces multiple toxic reactions including nystagmus, ataxia, dysarthria, and drowsiness. Kutt et al. (1964a) demonstrated that these toxic reactions are dose related; as the blood concentration of diphenylhydantoin rises, the severity of the toxic reaction increases progressively from nystagmus to ataxia to mental changes. To generalize from this and many other similar examples, variation among individuals in drug responsiveness, alluded to earlier as a major therapeutic problem, seems to arise not from differences among individuals in the nature of their physiologic or toxic receptor sites for drugs, but rather from differences among individuals in rates of drug clearance

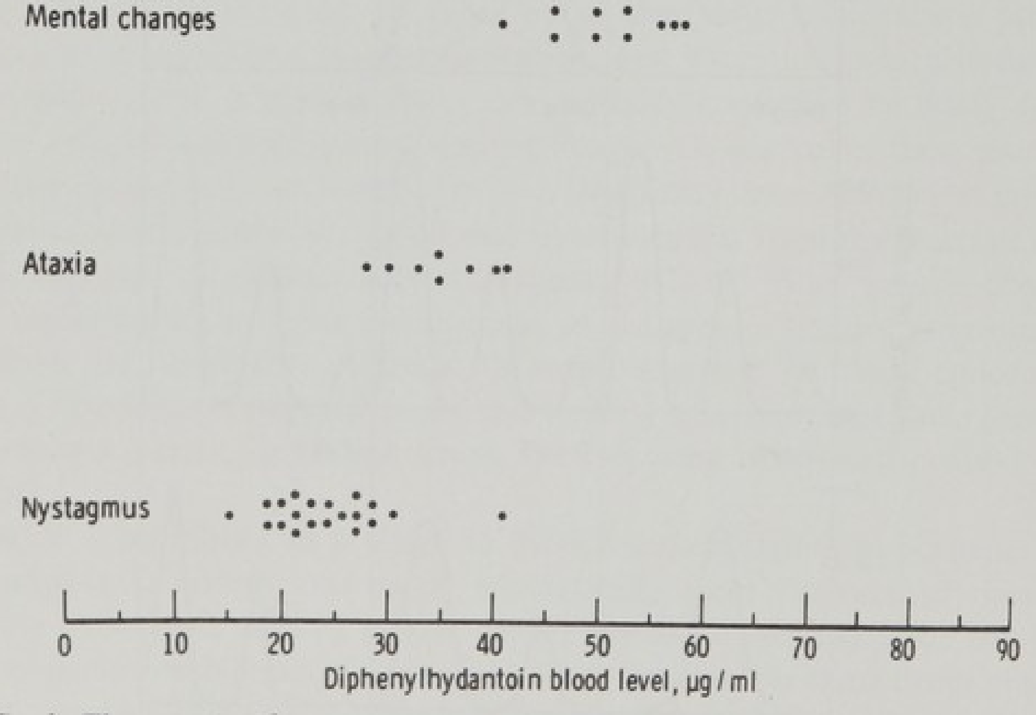


FIG. 3–The onset of nystagmus, ataxia, and mental changes in relationship to diphenylhydantoin blood concentrations, reproduced from Kutt et al. (1964a).

from the blood. Uniformity in the type of diphenylhydantoin toxicity produced by a given blood concentration is impressive in Fig. 3, but the question of how much diphenylhydantoin each individual shown in the figure required to attain a given blood concentration is not answered. It has been shown that significant differences exist among normal individuals in their capacity to clear diphenylhydantoin from blood (Glazko et al., 1969; Arnold and Gerber, 1970). Thus, if the same dose of diphenylhydantoin were administered by weight to normal unrelated subjects, they would exhibit markedly different diphenylhydantoin blood concentrations.

VARIATIONS AMONG INDIVIDUALS IN RATE OF DRUG ELIMINATION FROM HUMAN PLASMA

During the past 6 years, we have attempted to assess the relative contributions of genetic and environmental factors to large differences among individuals in rates of clearance of commonly used drugs. The results are of interest for both theoretical and practical reasons. If drug clearance from blood were a genetically controlled, highly reproducible trait, then it seemed feasible to type subjects for these rates and to administer drugs accordingly, thereby avoiding the extremes of undertreatment and toxicity in individuals with very fast or very slow drug-clearing capacity, respectively. Numerous environmental factors, such as exposure to inducing agents, degree of health or illness, and hormonal or nutritional status, are known to alter the rates at which humans metabolize certain drugs. Several drugs such as phenylbutazone enhance their own metabolism (Conney, 1967). In mice, responsiveness to a drug such as hexobarbital differs according to age, sex, litter, painful stimuli, ambient temperature, degree of crowding, time of day of drug administration, and type of bedding (Vesell, 1968). Such experiments would imply that in man a large component in the causation of variations among individuals in drug metabolism would be environmental.

The environmental and heritable components of individual variations in rates of drug metabolism can be determined through the use of human twins. Galton developed and introduced this use of twins in 1875. The approach has the virtue of simplicity in experimental design and execution, although it contains some important, rarely examined assumptions about environmental equality among identical and fraternal twins. By applying the twin method, we could estimate to what extent individual variations in drug metabolism were genetically controlled, reproducible, and of predictive value in the determination of individually optimum doses of drugs. Should extensive individual variations in drug response be maintained by genetic factors, then the rates at which patients metabolize drugs could be employed therapeutically to adjust doses of drugs prior to chronic administration. On the other hand, if rates of drug elimination in a given indi-

vidual or groups were predominantly under environmental control, then they would be expected to fluctuate extensively, depending on environmental alterations, and therefore would not be anticipated to constitute sufficiently stable values upon which to base long-term dosage.

In a series of studies conducted over the past 6 years, normal, adult, Caucasian twins living in the Washington, D.C., area were given single oral doses of various drugs to quantitate the genetic and environmental components of large individual variations in rates of drug elimination from plasma (Vesell and Page, 1968a, 1968b, 1968c; Cascorbi et al., 1971; Vesell et al., 1971a; Vesell et al., 1971d).

The environments of the volunteer twins were ascertained and during the course of the investigation were maintained unchanged from their usual patterns. Although no therapeutic agents were administered for 1 month preceding the study, this single limitation, imposed because of our goal to determine in the uninduced state the extent of and mechanisms responsible for individual variations in rates of drug elimination, did not represent much of a change for these volunteers. None had been on chronic medication. Many commonly encountered therapeutic agents enhance rates of drug elimination by inducing hepatic microsomal drug-metabolizing enzymes (Conney, 1967), so that to accomplish our purpose the twins could not ingest such drugs either during or for a short period before our investigation.

Because the environments prevalent in many large American cities contain compounds capable of altering rates of drug metabolism (Conney, 1967), differential individual exposure to such compounds as chlorinated hydrocarbons and insecticides was expected to produce an appreciable environmental contribution to large individual differences in rates of drug elimination from plasma. To assess the full extent of this environmental component, subjects should not be hospitalized either preceding or during the study. Therefore, the twins were permitted their customary range of activity at home and at work. All twins spent their working day apart, and only 2 pairs of the identical and 2 pairs of the fraternal twins lived in the same house so that most ate their noon and evening meals in different places.

An important aspect of this investigation concerned selection of drugs and the methods of administering them. The drugs chosen were phenylbutazone, antipyrine, bishydroxycoumarin, and ethanol because in man these agents are handled almost exclusively by biotransformation rather than by excretion of the unaltered parent drug. Phenylbutazone (Burns et al., 1953) and bishydroxycoumarin (Weiner, 1950) are avidly bound to plasma proteins; however, antipyrine and ethanol binding to albumin is negligible (Soberman et al., 1949). Administration of multiple doses of phenylbutazone (Conney, 1967) and antipyrine (Breckenridge and Orme, 1971) alters rates of drug metabolism, and bishydroxycoumarin is poorly absorbed from the gastrointestinal tract after

multiple doses (Weiner et al., 1950; O'Reilly and Aggeler, 1970) so that we were reluctant to employ the method of steady-state blood levels in our studies. Sjöqvist and his colleagues effectively used the steady-state method of repeated drug administration in studies on nortriptyline (Hammer et al., 1969), which is not an inducing agent, but which presents problems in gastrointestinal absorption and redistribution that make the steady-state method more suitable. However, we were obliged to measure plasma half-lives of the drugs we selected after only a single oral dose because chronic administration of these agents produces induction of the hepatic microsomal drug-metabolizing enzymes.

In our study, the volunteers were normal, adult, Caucasian twins who were typed for approximately 30 blood group antigens to document the nature of their twinship. At 9:00 a.m. each volunteer received a single oral dose of phenylbutazone (6 mg/kg), several months later a single oral dose of antipyrine (18 mg/kg), and several months later a single oral dose of bishydroxycoumarin (4 mg/kg). Blood specimens, drawn at regular intervals after drug ingestion, were analyzed for drug concentration in plasma and the values plotted as shown for phenylbutazone in Fig. 4 (Vesell and Page, 1968a). These curves illustrate typical examples of rates of phenylbutazone elimination from the plasma of identical and fraternal twins. Similar curves for identical and fraternal twins were obtained after administration of antipyrine (Vesell and Page, 1968b) and bishydroxycoumarin (Vesell and Page, 1968c). The half-lives of these three drugs were determined from such curves and are given in Table 2.

The half-life of ethanol was determined in the plasma of these twins after each twin received a single oral dose of 95% ethanol (1 ml/kg) at 9:00 a.m. (Vesell et al., 1971a). Plasma levels were estimated by gas chromatography (Goldbaum, 1964).

Genetic Control of Variations in Drug Clearance

For each of these four compounds intratwin differences in half-life are appreciably greater in fraternal than identical twins. Therefore, it can be concluded that genetic rather than environmental factors maintain large individual differences in rates of elimination of phenylbutazone, antipyrine, bishydroxycoumarin, and ethanol. Application of the following formula described by Neel and Schull (1954) and Osborne and De George (1959) allowed estimation of the contribution of heredity to large individual variations in the plasma half-lives of these drugs:

$$\frac{\text{(Variance within pairs of fraternal twins)} - \text{(Variance within pairs of identical twins)}}{\text{(Variance within pairs of fraternal twins)}}$$

This formula permits a range of values from 0, indicating negligible hereditary and complete environmental control, to 1, indicating virtually complete heredi-

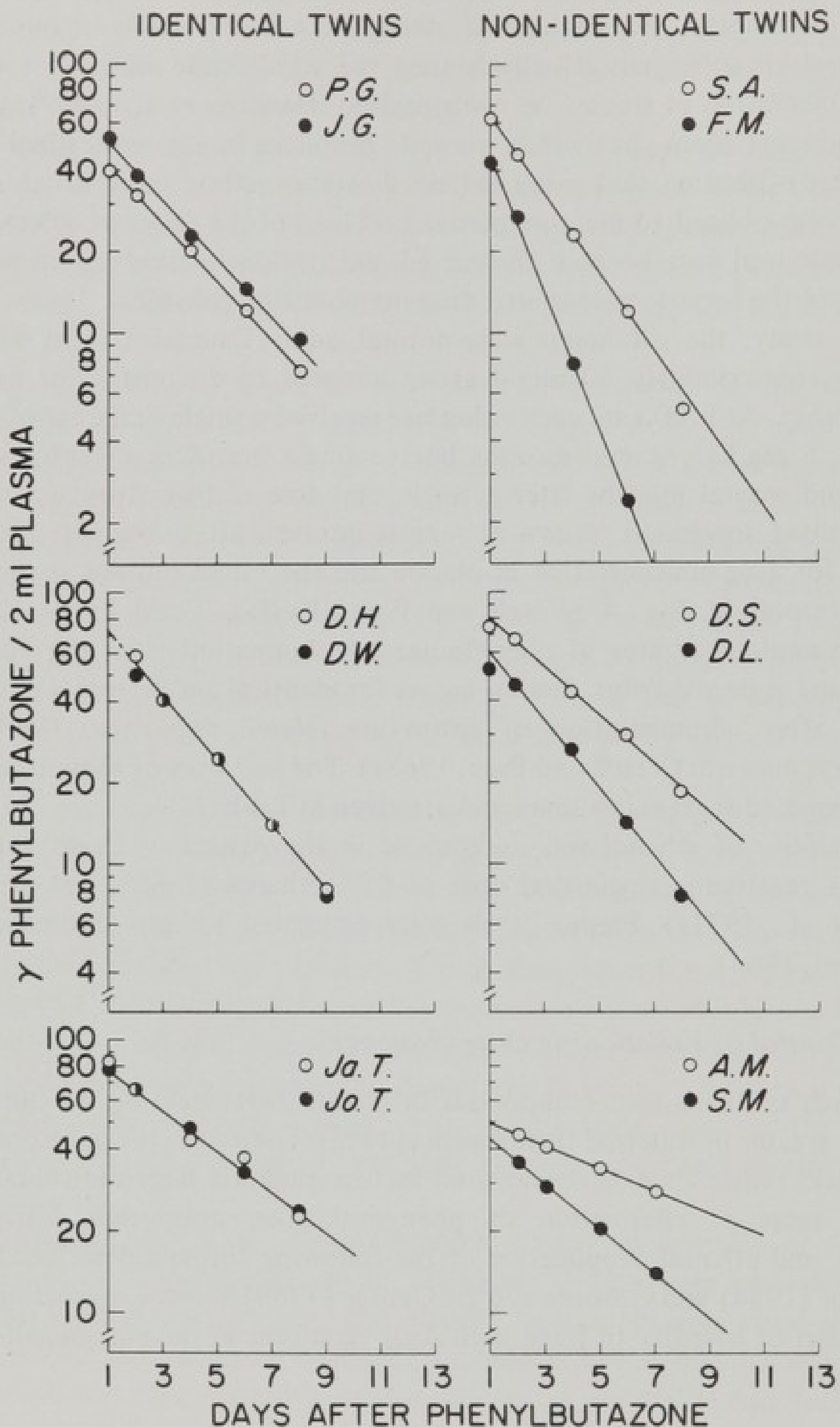


FIG. 4.–Decline of phenylbutazone in the plasma of 3 sets of identical twins (left) and 3 sets of fraternal twins (right). The log of the phenylbutazone concentration in 2 ml of plasma is shown at intervals after a single oral dose of 6 mg/kg, reproduced from Vesell and Page (1968a).

TABLE 2.–*Bishydroxycoumarin, Antipyrine, and Phenylbutazone Half-Lives with Smoking and Coffee History in 28 Twins**

Twin	Age, sex		Half-life			Smoking, pack/day	Coffee, cups/day
			Bishydroxy-coumarin, hr	Antipyrine, hr	Phenylbutazone, days		
Identical Twins							
Ho. M.	48,	M	25.0	11.3	1.9	0.5	2
Ho. M.	48,	M	25.0	11.3	2.1	1	3
D. T.	43,	F	55.5	10.3	2.8	0	5-6
V. W.	43,	F	55.5	9.6	2.9	2	8-10
J. G.	22,	M	36.0	11.5	2.8	1	1-2
P. G.	22,	M	34.0	11.5	2.8	1	1-2
Ja. T.	44,	M	74.0	14.9	4.0	0	6
Ja. T.	44,	M	72.0	14.9	4.0	0	2-3
C. J.	55,	F	41.0	6.9	3.2	0	2
F. J.	55,	F	42.5	7.1	2.9	0	2
Ge. L.	45,	M	72.0	12.3	3.9	0	4
Gu. L.	45,	M	69.0	12.8	4.1	0	4
D. H.	26,	F	46.0	11.0	2.6	0	0-1
D. W.	26,	F	44.0	11.0	2.6	0	3-4
Fraternal Twins							
A. M.	21,	F	45.0	15.1	7.3	1.5	2
S. M.	21,	M	22.0	6.3	3.6	0	0
D. L.	36,	F	46.5	7.2	2.3	0	2-3
D. S.	36,	F	51.0	15.0	3.3	2	3-4
S. A.	33,	F	34.5	5.1	2.1	1	2
F. M.	33,	F	27.5	12.5	1.2	0.5	2
Ja. H.	24,	F	7.0	12.0	2.6	0	10-15
Je. H.	24,	F	19.0	6.0	2.3	1.5	10
F. D.	48,	M	24.5	14.7	2.8	0	1
P. D.	48,	M	38.0	9.3	3.5	1.5	8
L. D.	21,	F	67.0	8.2	2.9	1	6
L. W.	21,	F	72.0	6.9	3.0	1	2-3
E. K.	31,	F	40.5	7.7	1.9	0	0
R. K.	31,	M	35.0	7.3	2.1	1	0

The difference between identical and fraternal twins in intrapair variance is significant: $P < 0.005$ ($F = 36.0, N_1 = N_0 = 7$).

* From Vesell and Page (1968c).

tary influence. The contribution of heredity to variations in the half-life of phenylbutazone, antipyrine, bishydroxycoumarin, and ethanol was calculated to be 0.99, 0.98, 0.97, and 0.99, respectively. In these investigations we obtained intraclass correlation coefficients not very far from theoretical expectation on

the basis of complete genetic control, according to which fraternal twins, having in common approximately half their total number of genes, should exhibit a value of 0.5, whereas identical twins should have a value of 1. The intraclass correlation coefficients of identical twins for phenylbutazone, antipyrine, bishydroxycoumarin, and ethanol were 0.83, 0.85, 0.85, and 0.82, respectively, whereas in fraternal twins the values for these drugs were 0.33, 0.47, 0.66, and 0.38, respectively (Vesell and Page, 1968a, 1968b, 1968c; Vesell et al., 1971a). Thus, in normal subjects not receiving other therapeutic agents large individual differences in rates of elimination of these drugs from plasma are surprisingly free of environmental influence. Repeated half-life determinations revealed that nonmedicated, normal subjects have very reproducible plasma half-lives for these drugs. Phenylbutazone (Burns et al., 1953) and bishydroxycoumarin (Weiner et al., 1950) are 98% bound to plasma proteins, so that differences among individuals in rates of plasma clearance of these drugs might possibly involve binding to albumin. Antipyrine and ethanol, on the other hand, are not appreciably bound to plasma proteins (Soberman et al., 1949). Therefore, for antipyrine and ethanol, if not also for phenylbutazone and bishydroxycoumarin, variations in plasma half-life arise from genetic differences that involve metabolism rather than distribution. The ranges for the plasma half-lives of ethanol, antipyrine, phenylbutazone, and bishydroxycoumarin of 2-fold, 3-fold, 6-fold, and 10-fold, respectively, among the 28 individuals in our study indicate large variations among these individuals in rates of plasma clearance.

Another study of these same twins revealed almost fourfold variations in metabolism of a single intravenous dose of 3.4 mg of radioactive halothane (Cascorbi et al., 1971). Large individual differences in halothane metabolism were demonstrated to be predominantly controlled by genetic factors (Cascorbi et al., 1971), although the corrected value of 0.88 and the uncorrected value of 0.63 for the contribution of heredity to individual differences in rates of halothane metabolism indicate a larger environmental component for this drug than was observed for ethanol, phenylbutazone, antipyrine, or bishydroxycoumarin. Two reasons that could account for the greater environmental influence over variations in halothane than in phenylbutazone, antipyrine, or bishydroxycoumarin metabolism include the possibility of its control by a different enzyme system more susceptible to induction by commonly encountered environmental substances or alternatively the fact that halothane is metabolized to a much smaller extent than the other drugs we investigated. The percent of the administered dose of halothane metabolized ranges in these 20 subjects from only 2.7 to 11.4. When such a small fraction of an administered dose is metabolized, environmental contributions to variations in biotransformation could play a proportionately larger role. For halothane, the intraclass correlation coefficient for identical twins was 0.52 and for fraternal twins 0.36, respectively.

Table 3 shows heritability of variations in the metabolism of these drugs as

TABLE 3.–*Heritability of Variations in Drug Metabolism of Twins Utilizing Different Methods of Data Analysis*

	Antipyrine	Phenyl-butazone	Bishydroxy-coumarin	Ethanol	Halothane
$\frac{V_F - V_I}{V_F}$	0.98	0.99	0.97	0.98	0.88
r_I	0.85	0.83	0.85	0.82	0.52
r_F	0.47	0.33	0.66	0.38	0.36
$\frac{r_I - r_F}{1 - r_F}$	0.72	0.75	0.56	0.71	0.25
$2(r_I - r_F)$	0.76	1.00	0.38	0.88	0.32

r = intraclass correlation coefficient.

calculated by several other methods. Other methods were employed because the technique utilized in the preceding estimates assumes that environmental differences between twinships are negligible; and, therefore, these calculations are too high to the extent that such differential environmental factors operate. Falconer (1960) approached the problem by partitioning variance into several genetic and environmental components; although this approach can be utilized in the family studies (Burt and Howard, 1956), it cannot be applied to twin data. Halothane shows a very small genetic component of control by the Holzinger (1929) index

$$\frac{r_I - r_F}{1 - r_F}$$

or by the method of Falconer (1960), modified by using values of V_A/V_P (variance due to additive gene effects and phenotypic factors, respectively) provided from the family study of Whittaker and Price Evans (1970). The estimation of hereditary control of a trait, according to Falconer (1960), is based on the proportion of the phenotypic variance contributed by the two genetic components of variance, the so-called additive and dominance components of variance:

$$H = 2(r_I - r_F) = \frac{V_A + 1.5\ V_D}{V_P}$$

A twin study performed in Stockholm on variations in steady-state blood concentrations of nortriptyline in otherwise nonmedicated, nonhospitalized twins showed appreciably smaller intratwin differences in identical than in fraternal twins (Alexanderson et al., 1969). These marked differences between identical and fraternal twins in steady-state blood levels of nortriptyline confirmed our conclusions from twin studies with phenylbutazone, antipyrine, bishydroxycoumarin, ethanol, and halothane. A value of 0.98 for heritability of variations among individuals in nortriptyline metabolism was calculated from the twin data of Alexanderson et al. (1969).

Recently, Whittaker and Price Evans (1970) performed a family study to assess the genetic contribution to variability among individuals in phenylbutazone metabolism. They concluded that variability among individuals in phenylbutazone metabolism was under polygenic control. Previously, a similar conclusion was reached by Motulsky (1964) from a family study on variability in the plasma half-lives of bishydroxycoumarin after a single oral dose. Whittaker and Price Evans (1970) obtained a normal distribution of phenylbutazone half-lives in plasma after correcting for height and also after administering a 3-day course of phenobarbital to "render the environment more uniform." A significant regression of mean offspring value on midparent value indicated to Whittaker and Price Evans that approximately 65% of the observed phenotypic variance was caused by the additive effects of genes. These results agree closely with those from our earlier study on phenylbutazone metabolism in twins (Vesell and Page, 1968a) if V_D (variance due to the effect of dominance) is not neglected in the following formula derived from Falconer (1960):

$$H = \frac{\frac{1}{2} V_A + \frac{3}{4} V_D}{\frac{1}{2} V_A + \frac{3}{4} V_D + V_E} = \frac{V_F - V_I}{V_F}$$

where V_A = the variance due to additive effects of genes controlling the trait, V_E = the variance due to environment, F = fraternal twins, and I = identical twins. Whittaker and Price Evans (1970) state that V_D is too small to be significant, although they admit that V_{Ec} is probably large. ($V_E = V_{Ew} + V_{Ec}$, where V_{Ew} and V_{Ec} are the within-twin-pair variance and the common variance between twin pairs, respectively.) Neither V_D nor V_{Ec} was measured in their family study. In other studies of polygenically controlled traits in man, V_D is small but not negligible. For example, in their classic study of height and intelligence, Burt and Howard (1956) reported a value of 0.16 and 0.17 for the contribution of V_D to height and intelligence, respectively. If their value for V_D of 0.16 is utilized as an estimate of V_D in calculating the family data of Whittaker and Price Evans (1970)—and it seems more reasonable to use this hypothetical value than to disregard V_D completely—there is good agreement between the results of the family study of Whittaker and Price Evans ($H = 0.88$)

and our twin data on phenylbutazone ($H = 0.75$ or 1.00) (Table 3). Since these values are close to the estimate (0.99) based on the formula for heritability that we employed

$$H = \frac{V_F - V_I}{V_F}$$

we may conclude that differential environmental factors operating between twinships in our investigation were quite small. Another recent study in two extensive Swedish pedigrees with high steady-state plasma concentrations of nortriptyline suggested that the appreciable individual differences in the steady-state plasma concentrations of this drug were polygenically controlled (Åsberg et al., 1971). Thus far both the family studies and the twin data have agreed in their conclusions that large differences among healthy, nonmedicated volunteers in rates of drug metabolism are primarily controlled by genetic factors.

The use of twins has lost favor in human genetics; although the defects inherent in twin studies have been repeatedly emphasized, their advantages in investigating variations among individuals in rates of drug metabolism have not previously been described. Even the assumption that "monozygotic human twins have identical inheritance" has been challenged by Storrs and Williams (1968) on the basis of large differences in 20 parameters among newborn monozygotic quadruplet armadillos. The twin method does suffer from several disadvantages including its inability to establish conclusively the mode of inheritance of a genetically controlled trait and its assumption of an environmental equality in all subjects, identical as well as fraternal twins. It has been argued that in man this latter assumption is invalid because identical twins make more similar choices and have more similar tastes than fraternal twins in eating, drinking, and even toothpaste. However, in our studies the identical twins lived for the most part in different homes and ate different meals so that these environmental similarities that might possibly affect drug metabolism seemed superficially no greater among the identical than among the fraternal twins. Each of our subjects was over 21 years of age and had established for himself a life pattern relatively independent of and different from that of his twin.

Some reviewers maintain that "while twin studies can define the heritable nature of an unusual response to a drug, they are of little value in elucidating the genetics of a disorder further" (Cohen and Weber, 1972). However, Fig. 5 demonstrates that twin studies can provide important clues toward the elucidation of the mode of inheritance of pharmacogenetic variation. Figure 5 shows a trimodal distribution curve strongly suggesting single-factor inheritance of variations among only 10 sets of twins in response to isonizid (Bönicke and Lisboa, 1957). By way of contrast, another twin study, also of a very small number of twins, reveals a unimodal distribution of antipyrine half-lives (Vesell and Page,

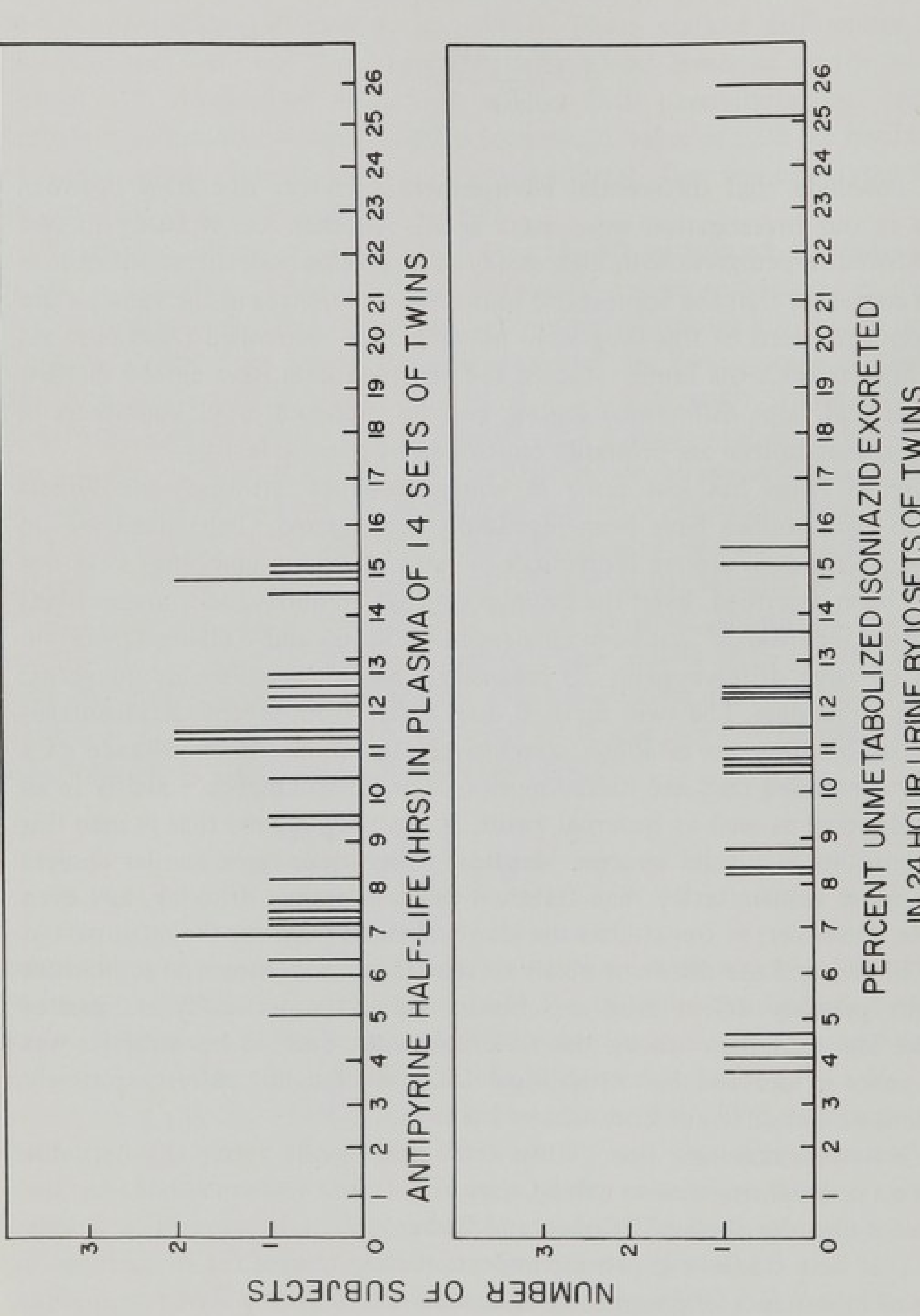


FIG. 5.–Distribution curves of response in two twin studies. The lower distribution curve is for the 24-hour excretion of isoniazid in the urine of 5 sets of identical and 5 sets of fraternal twins (Bönicke and Lisboa, 1957). The upper distribution curve is for antipyrine half-lives in 7 sets of identical twins and 7 sets of fraternal twins (Vesell and Page, 1968b).

1968b) suggesting polygenic control of these variations (Fig. 5). Thus, even for the very small number of twins illustrated in Fig. 5, construction of a distribution curve clearly helps to suggest the mode of inheritance of genetically controlled variations in response to drugs.

Twin studies can be a particularly profitable initial step in identifying the relative genetic and environmental contributions to a new trait. Then, the construction of a distribution curve of these twins can provide a useful hint as to whether such variations are transmitted polygenically or as simple single factors. It should be emphasized here that for a rare autosomal recessive trait, a unimodal curve might be obtained if too few individuals were tested. This eventuality could lead to an erroneous interpretation of polygenic control. Twin studies alone cannot conclusively establish the mode of inheritance of a pharmacogenetic entity, but through distribution curves of drug response, twin data can be utilized more than they have been previously. Family studies should, as the third step in the genetic delineation of a new trait, then be performed at the extremes of this distribution curve where pedigrees would be most likely to prove genetically informative.

In the evaluation of variations in drug metabolism, twin studies enjoy several distinct advantages over family studies and have been utilized on rare occasions as subjects for investigations of the genetic component of individual variations in drug metabolism (Bönicke and Lisboa, 1957; Kappas and Gallagher, 1960). Twins are by definition age corrected, and dizygotic twins of the same sex can easily be selected. As studies in rodents have shown (Vesell, 1968) and as studies in man have suggested (O'Malley et al., 1971), rates of drug metabolism change with age and sex. Thus the genetic analysis of data on drug metabolism from family studies is complicated by incorporation of variations in rates of drug metabolism from differences in age and sex, two factors readily eliminated in twin studies. Furthermore, differences in the environment of children, parents, and grandparents with respect to exposure to certain environmental compounds capable of inducing or inhibiting the hepatic microsomal drug-metabolizing enzymes must be considered a source of possible variation in family studies. Such environmental influences on drug metabolism arising from common exposure in the same household to inducers or inhibitors of drug-metabolizing enzymes could explain why, in the family study of Whittaker and Price Evans (1970), there was a correlation in phenylbutazone metabolism between husbands and wives before phenobarbital administration.

Environmental Influences on Variations in Drug Clearance

We have sought possible correlations in rates of metabolism of different drugs in single individuals. If such correlations existed, then drugs could be grouped into categories such that once the individual's rate of metabolism of one drug in a category were ascertained, his rate of handling all the others in that category

could be readily calculated. Typing individuals for their rates of drug metabolism would provide a new approach to reducing instances of drug toxicity and undertreatment. Therefore, attempts were made to correlate rates of metabolism of phenylbutazone, antipyrine, bishydroxycoumarin, and ethanol. The only correlation between the plasma half-lives of these drugs after a single oral dose of each was between rates of metabolism of phenylbutazone and bishydroxycoumarin (Vesell and Page, 1968c). This correlation was obtained using the method of Bartlett (1949). In an individual, correlation between rates of phenylbutazone and bishydroxycoumarin metabolism might be due to the avid and almost complete binding of these drugs by albumin. An alternative explanation would depend on the existence of similar enzymatic steps or a common rate-limiting step in the biotransformation of both drugs. The possibility emerges from this work that within an individual the rates of decay of other drugs may also be correlated; and, in support of this hypothesis, recent work by Hammer et al. (1969) established correlation within a given individual of the rates of metabolism of desmethylimipramine, nortriptyline, and oxyphenylbutazone. Furthermore, after chronic dosage, antipyrine metabolism in an individual does correlate with phenylbutazone metabolism (Davies and Thorgeirsson, 1971), although these workers were also unable to correlate rates of metabolism of these two drugs after only a single oral dose of each.

Multiple, diverse environmental conditions are known to affect rates of bishydroxycoumarin, diphenylhydantoin, or phenylbutazone metabolism; even the size of the dose and the extent and rapidity of gastrointestinal absorption alter the decay of these drugs (Vesell et al., 1971d). Of course, prior ingestion of substances that induce or inhibit drug-metabolizing enzymes located in liver microsomes will affect the metabolism of these, as well as most other, drugs. In addition to phenobarbital and diphenylhydantoin, several substances such as 3,4-benzpyrene, 3-methylcholanthrene, nicotine (Wenzel and Broadie, 1966), and caffeine (Mitoma et al., 1968), to which many individuals expose themselves, can accelerate drug metabolism, thereby shortening the plasma half-life of many therapeutic agents. Because benzpyrene hydroxylase activity was elevated in the placenta of smokers (Welch et al., 1969), a history of cigarette smoking and coffee ingestion was taken in our 28 twins (Table 2). However, these habits did not seem to correlate with the rate of bishydroxycoumarin metabolism; in fact, several instances of discordance occurred, such as in a set of identical twins with closely similar bishydroxycoumarin half-lives, one of whom smoked two packages of cigarettes a day, whereas the other did not smoke. Since only 2 of the 7 sets of identical twins lived in the same household, the close resemblance between identical twins in phenylbutazone, antipyrine, and bishydroxycoumarin half-lives does not seem attributable to such environmental factors as exposure to the same inducing agents, which would be expected to operate on individuals

sharing the same home and meals. Large intrapair differences in drug half-life between two sets of fraternal twins who lived together further support this conclusion.

Compounds capable of shortening either their own duration of action or that of other drugs administered simultaneously (Yaffe et al., 1966; Ramboer et al., 1969) have been utilized clinically; and, therefore, the magnitude of differences among individuals in responsiveness to such inducing agents becomes a therapeutically important problem. The simplest assumption would be that if comparable blood levels of an inducing agent were obtained, then the activity of the drug-metabolizing enzymes would rise to similar extents. However, this was not the case; and for phenobarbital, the inducing agent most commonly employed therapeutically in man, large, genetically determined differences exist in the inductive response, even in the face of similar blood concentrations of phenobarbital (Vesell and Page, 1969). In 4 sets of identical and 4 sets of fraternal twins, antipyrine half-lives were measured before and after 2 weeks of sodium phenobarbital administered in a daily dose of 2 mg/kg. Intrapair differences in the extent to which phenobarbital altered antipyrine half-lives were significantly greater in fraternal than in identical twins, as shown in Table 4 (Vesell and Page, 1969). The contribution of heredity to variations in the extent of phenobarbital-induced reduction in plasma antipyrine half-life, and hence to variations in the induction of hepatic, microsomal drug-metabolizing enzymes by phenobarbital, was calculated to be 99% by the formula for H described above.

In these studies phenobarbital decreased the standard deviation of the mean antipyrine half-life by more than twofold and the variation in antipyrine half-life from 2.8-fold to 1.8-fold (Table 4). These results suggest that if extensive individual differences in drug metabolism produce therapeutic problems, relatively innocuous inducing agents might be administered to minimize such variability.

With respect to this suggestion, a direct relationship occurred between initial antipyrine half-life and the percent shortening of antipyrine half-life produced by phenobarbital administration; the longer the initial antipyrine half-life, the greater the reduction caused by phenobarbital treatment (Vesell and Page, 1969). This relationship permits prediction of the extent to which phenobarbital will reduce initial antipyrine half-life; since phenobarbital shortens the antipyrine half-life of slow metabolizers more than it shortens the antipyrine half-life of fast metabolizers, use of phenobarbital to reduce toxic blood levels of various drugs would appear to aid preferentially those individuals most in need of such therapy, namely, the slow metabolizers. Because the genes controlling variations among individuals in inducibility after phenobarbital may be different from the genes controlling variations among relatively uninduced individuals in the metabolism of drugs such as phenylbutazone and antipyrine, it is difficult to interpret results of a study which mixes these two phenomena by measuring

TABLE 4.–*Response of Plasma Antipyrine Half-Life to Phenobarbital Administration with Smoking, Coffee, Tea, and Alcohol History in 16 Twins**

Twin	Age, sex	Plasma antipyrine half-life: Before pheno-barbital, hr	Plasma antipyrine half-life: After pheno-barbital, hr	Decrease in half-life produced by pheno-barbital, %	Percentage difference between sibs in response to pheno-barbital	Plasma pheno-barbital levels at 156 hr	Plasma pheno-barbital levels at 212 hr	Smoking, pack/day	Coffee, cup/day	Tea, cup/day	Alcohol: Beer, bottles/day	Alcohol: Wine, glasses/day	Alcohol: Hard liquor, oz/day
Identical Twins													
Dan E.	22, M	13.6	9.6	29.4	0	20.0	23.0	0	2	0	¼	0	¼
Dav. E.	22, M	13.6	9.6	29.4		20.0	23.2	¼	0	2	¼	0	¼
A. M.	35, F	8.0	6.3	21.2	0	17.0	18.0	½	0	3	0	0	0
B. Z.	35, F	8.0	6.3	21.2		15.0	16.0	2	6-7	0	0	0	1
Bar. J	23, F	18.2	8.4	53.8	0	16.8	17.1	0	0	0	0	0	0
Bev. J.	23, F	18.2	8.4	53.8		17.9	25.0	0	0	0	0	0	0
B. F.	26, F	10.8	7.3	32.4	2.6	16.0	23.4	0	0	0	0	0	0
B. J.	26, F	11.4	7.4	35.0		17.4	24.4	0	0	1	0	0	¼
Fraternal Twins													
F. D.	49, M	12.0	10.3	14.2	14.2	14.8	19.4	0	1	2	0	0	0
P. D.	49, M	9.3	9.3	0		15.2	18.3	1½	5	0	0	0	1
C. K.	49, M	17.5	5.5	68.6	8.6	–	23.0	1½	2	0	1	0	18
N. R.	49, F	14.5	5.8	60.0		–	19.0	1½	4	0	0	0	0
H. H.	47, F	12.3	9.2	25.2	9.8	17.8	16.6	2	2	0	½	0	½
P. M.	47, F	6.5	5.5	15.4		16.0	20.8	1½	2	0	½	0	0
E. W.	54, F	15.0	6.9	54.0	30.7	20.0	29.0	0	4	0	0	0	½
E. E.	54, F	9.0	6.9	23.3		20.0	30.0	1	2	2	0	0	¼

* From Vesell and Page (1969).

variations among individuals in the metabolism of a drug such as phenylbutazone only after all the subjects have been induced by phenobarbital (Whittaker and Price Evans, 1970).

It is known that many commonly administered drugs enhance the activity of hepatic microsomal drug-metabolizing enzymes. However, inhibition of drug metabolism in man is less well appreciated, although the clinical consequences of drug inhibition may be more severe than the sequelae of induction. Very few inhibitors of drug metabolism have been identified. Some of these are methylphenidate (Garrettson et al., 1969), oxyphenylbutazone (Weiner et al., 1965), methandrostenolone (Weiner et al., 1965), and phenyramidol (Solomon and Schrogie, 1966). Inhibition of drug metabolism would appear to be much less frequent an occurrence than induction. Therefore, it was a surprise to discover that several randomly selected, commonly employed drugs (the tricyclic antidepressant nortriptyline; the xanthine oxidase inhibitor allopurinol, used in the treatment of gout; and disulfiram, used to inhibit alcohol dehydrogenase in alcoholics) when tested in man were observed to impair drug metabolism (Vesell et al., 1970; Vesell et al., 1971b).

We employed as subjects healthy, male, Caucasian medical students not taking medication at the time of, or for 1 month preceding, the study. Each volunteer served as his own control, the control being represented by his plasma antipyrine or bishydroxycoumarin half-life after a single oral dose of antipyrine (18 mg/kg) or bishydroxycoumarin (4 mg/kg). Each volunteer then received either nortriptyline (0.2 mg/kg three times a day orally) for 8 days, or allopurinol (2.5 mg/kg twice a day orally) for 2 weeks, or disulfiram (7 mg/kg daily) for 4 days. Twenty-four hours after the last dose of nortriptyline, allopurinol, or disulfiram, plasma antipyrine or bishydroxycoumarin half-lives were redetermined following another oral dose of either antipyrine or bishydroxycoumarin. Chronic administration of nortriptyline, allopurinol, and disulfiram prolonged the plasma half-lives of antipyrine and bishydroxycoumarin, but for each drug marked differences occurred among individuals in the extent of this prolongation. For nortriptyline and allopurinol fivefold variations among individuals (Vesell et al., 1970) and for disulfiram almost threefold variations among individuals (Vesell et al., 1971b) were observed in the extent of retardation of antipyrine or bishydroxycoumarin half-lives. Although no family or twin studies were performed to determine the relative genetic and environmental components of this variability, it is known that on the daily doses of allopurinol we administered, only small individual variations in allopurinol plasma concentrations occur (Elion, personal communication). Therefore, it appears that, at least for allopurinol, large differences among individuals in the degree of retardation of drug metabolism are not attributable to differences among subjects in the blood concentrations of these drugs.

An interesting example of drug interaction producing retardation of drug

metabolism involves L-dopa and the dopa decarboxylase inhibitor, L-α-methyldopa (Vesell et al., 1971c). Neither L-dopa administered alone for 2 weeks in a dose reaching 3 g/day on days 11, 12, 13, and 14 nor the dopa decarboxylase inhibitor administered alone in a dose of 50 mg three times a day significantly altered rates of antipyrine metabolism. However, in combination, the dopa decarboxylase inhibitor administered simultaneously with lower doses of L-dopa significantly prolonged antipyrine metabolism. On this combination 5 of the 6 subjects showed retardation of plasma antipyrine half-lives. More than a twofold range occurred in the extent of this inhibition. It is interesting from the viewpoint of individual variations that, in 2 of the 6 subjects, receiving the dopa decarboxylase inhibitor alone prolonged their antipyrine metabolism to an appreciable extent. Although no blood levels of L-dopa were measured in this study, we interpret the results to indicate that the dopa decarboxylase inhibitor elevated the blood concentrations of the simultaneously administered L-dopa to levels above those attained when L-dopa was administered alone and that these higher blood concentrations of L-dopa inhibited antipyrine metabolism. This interpretation is justified by previous work demonstrating elevated blood concentrations of L-dopa after administration of dopa decarboxylase inhibitors (Pletscher and Bartholini, 1971). Again, appreciable variation occurred in the extent to which L-dopa prolonged antipyrine half-life even in this small study of 6 patients receiving both L-dopa and L-α-methyldopa.

There have been few investigations in man of the possible effects exerted by diurnal variations, age, and sex on drug metabolism. A recent study describes 45% and 29% prolongation of antipyrine and phenylbutazone half-lives, respectively, in geriatric patients as compared with young controls (O'Malley et al., 1971). Furthermore, these data indicated a sex difference in antipyrine metabolism, the mean half-life being 30% longer in males than in females.

GENETIC CONDITIONS PROBABLY TRANSMITTED AS SINGLE FACTORS ALTERING THE WAY THE BODY ACTS ON DRUGS

Acatalasia

Although no cases of acatalasia have been reported in the United States, the history of acatalasia illustrates how clinical acumen can lead to the discovery of pharmacogenetic disorders. In 1946 the Japanese otorhinolaryngologist Takahara discovered acatalasia after he completed surgery on an 11-year-old Japanese girl for a friable granulating tumor in the right nasal cavity and maxillary sinus (Wyngaarden and Howell, 1966). Takahara excised the necrotic areas and then applied hydrogen peroxide to sterilize the wound. In this girl the usual bubbles of oxygen, liberated by the action of catalase on hydrogen peroxide, did not occur and, furthermore, the color of the tissue darkened, turning black, presumably through oxidative denaturation of hemoglobin by the drug. Although Taka-

hara initially believed that silver nitrate had been used by accident, he observed the same bizarre response after rewashing the wound and applying hydrogen peroxide from a fresh bottle. Takahara then suspected that his patient lacked the enzyme catalase and demonstrated that this explanation was correct in a series of classic studies; the defect was shown by Takahara to be transmitted as an autosomal recessive trait (Takahara, 1952, 1954; Takahara et al., 1952; Takahara and Doi, 1958, 1959). His initial patient lacked catalase activity in her oral mucosa and erythrocytes; 3 of her 5 siblings also lacked the enzyme. As observed frequently in rare inborn errors of metabolism, consanguinity existed; the patient's parents were second cousins.

Because "acatalasemia" suggests restriction of the defect to blood, the term has been dropped in favor of "acatalasia" since the enzyme is deficient in such tissues as mucous membrane, skin, liver, muscle, and bone marrow. "Acatalasia" is not an entirely accurate designation either, because trace levels of catalase activity occur in certain patients for whom the term severe hypocatalasia seems more appropriate (Wyngaarden and Howell, 1966). Heterozygotes who generally exhibit values of catalase activity between those of the homozygous recessives and normal individuals would be classified as having "intermediate hypocatalasia." Further complications and heterogeneity are introduced by certain Japanese kindreds in which heterozygotes do not exhibit intermediate levels of catalase activity but rather values that overlap with the normal range (Hamilton and Neel, 1963).

Intensive screening for individuals with acatalasia was conducted and 38 cases in 17 families were gathered by 1959 (Takahara and Doi, 1958, 1959). Affected individuals were scattered throughout Japan in a fashion that suggested considerable geographic variation, with "pockets" where this normally rare gene might occur in frequencies as high as 12%. In other regions of Japan, much lower gene frequencies of approximately 0.3% were observed.

Mild, moderate, or severe expressions of acatalasia have been described (Takahara et al., 1960). The mild form of the disease is characterized by ulcers of the dental alveoli, whereas alveolar gangrene and atrophy occur in moderate types. In the severe form, recession of alveolar bone develops, with exposure of the necks of teeth, resulting eventually in their loss.

Studies of catalase acitivity in 66 members of 5 affected families revealed that males and females were equally affected and that the distribution of activity was trimodal, corresponding to three phenotypes designated acatalasemic, hypocatalasemic, and normal by Nishimura and associates (1959).

Yata reported a Korean case of acatalasia in 1959; this was the first example of a non-Japanese subject with the disorder to be described. Two years later Aebi and associates published the results of screening 73,661 blood samples from Swiss Army recruits; they discovered 3 individuals with acatalasia. All 3 were in excellent health and revealed none of the dental abnormalities character-

istic of the Japanese cases. Furthermore, unlike the Japanese cases, the Swiss "acatalasics" exhibited some residual catalase activity, possibly protecting them against the hydrogen peroxide formed by certain microorganisms that are thought to be responsible for the oral lesions.

A classification of the different types of acatalasia reported from several countries is shown in Table 5 from Aebi (1967a). At least several distinguishable forms of acatalasia probably exist, as indicated by differences in catalase activity, gene frequency, geographical distribution, and clinical manifestations. Different properties of the catalase molecule isolated from individuals with different forms of the disease (Shibata et al., 1967) also suggest genetic heterogeneity.

Initially, catalase molecules were reported to be identical with respect to enzymatic and antigenic properties in normal individuals and subjects with the Swiss type of acatalasia (Micheli and Aebi, 1965; Aebi et al., 1964). Later, however, the catalase from Swiss cases was demonstrated to differ from the catalase of normal individuals (Aebi, 1967a, 1967b). Electrophoretic differences between the catalases of normal and deficient individuals were detected after further purification of the enzyme. In addition, differences in pH and heat stabilities were established. Furthermore, normal catalase differed from the catalase of deficient subjects in sensitivity toward the inhibitors aminotriazole and azide. All these observations suggested that in Swiss families acatalasia was a structural, rather than a controller, gene mutation (Aebi, 1967b).

Catalase in human erythrocytes exists in electrophoretically distinguishable forms or isozymes (Price and Greenfield, 1954; Holmes and Masters, 1965; Nishimura et al., 1964; Thorup et al., 1964; Baur, 1963). Catalase isozymes all exhibit molecular weights of approximately 250,000. Further complication was introduced by the description of a minor component of catalase which, though reacting with rabbit antihuman catalase serum, lacks enzyme activity (Shibata et al., 1967). The minor inactive component with a molecular weight of approximately 60,000 (Shibata et al., 1967) may be a subunit or precursor of catalase, and other data suggest that bovine liver catalase is composed of three or four identical chains (Schroeder et al., 1964; Tanford and Lovrien, 1962). Possibly in acatalasics the minor inactive component may be structurally different from that in normocatalasics. Although one possibility is that such a structural abnormality might render the subunits unable to assemble into the polymeric form of the "apocatalase" molecule, an alternative explanation postulates that in acatalasics the precursor subunits are entirely normal, but a defect exists in a hypothetical "coupling" enzyme that may be required to join the subunits prior to addition of the prosthetic group (Shibata et al., 1967).

Slow Inactivation of Isoniazid

Synthesized in 1912 by Meyer and Mally, isoniazid or *l*-isonicotinylhydrazine was not discovered to exert a bacteriostatic effect on *Mycobacterium tubercu-*

TABLE 5.–*Cases of Acatalasia and Related Anomalies Reported in Literature Until 1965**

Type (year of detection)	Origin (no. of families)	Number of homozygotes (Hom) and heterozygotes (Het)	Residual catalase activity percentage (normal = 100)	Remarks
I (1947)	Japan (31) Korea (1)	Hom: 66 Het: > 100	Hom: 0-3.2 Het: 37-56	Incomplete recessive inheritance; oral gangrene (Takahara's disease) in ∿50% of homozygotes; activity: trimodal distribution curve (no overlap)
II (1959)	Japan (1) family 13 MI	Hom: 1 (male) Het: –	Hom: 3.2 Het: ∿ 100	Complete recessive inheritance (inolvement of modifier or suppressor genes?)
IIIa (1962)	Japan (1) kindred 29 OHH	Hom: 3 (Het: 17)	Hom: 0 (?) Het: > 56	Overlap between heterozygous carrier and normals (dual allelic control?)
IIIb (1961)	Switzerland (3) families V. B. and G.	Hom. 11 (Het: ∿ 30)	Hom: 0.1-1.3 Het: 15-85	Synthesis of two different types of catalase in heterozygotes (normal catalase + unstable variant), all homozygotes in good state of health
IV (1963)	Israel (1) Iranian born	Hom: 1 (male) Het: 15	Hom: 8 Het: 49-67	Combination with deficiency of G-6-PD; intolerance to fungicide
V (1963)	United States (1) Scandinavian and British extraction	Hom: 0 Het: 6	All: ∿ 100	Allocatalasia: synthesis of a variant catalase; activity and stability as normal catalase

* From Aebi (1967a).

losis until 1952 (Grunberg et al., 1952; Robitzek et al., 1952). Shortly thereafter, large variations were reported in the metabolism of isoniazid in man (Bönicke and Reif, 1953; Hughes, 1953; and Hughes et al., 1954, 1955). Studies of the excretory products of isoniazid revealed that all the drug appeared in the urine either as acetyl isoniazid, isonicotinic acid, unchanged isoniazid, or small amounts of other derivatives (Hughes et al., 1955). Although extensive variations occurred among individuals in the amount of free or acetylated urinary products, which were inversely related within every subject, each subject maintained his pattern of excretion unchanged during long-term therapy.

The isoniazid polymorphism might be expected to result in rapid inactivators' responding less favorably than slow inactivators to treatment. In accord with this prediction, Harris (1961b) reported that in 775 patients with pulmonary tuberculosis on standardized isoniazid regimens cavity closure and sputum conversion were generally noted earlier in slow than in rapid inactivators. However, after 6 months of treatment no clinically detectable differences were observed between slow and rapid phenotypes. If isoniazid is administered only once a week (Price Evans, 1968), then responses are worse in rapid than in slow inactivator patients with tuberculosis. Neither the slow nor the rapid acetylase genotype is apparently more liable to resistance to tubercle bacilli (Harris, 1961b) or reversion (Gow and Price Evans, 1964).

The major clinical problem associated with this polymorphism is polyneuritis, which does occur more frequently in slow inactivators. During isoniazid therapy polyneuritis occurred in 4 of 5 slow inactivators, but in only 2 of 10 rapid inactivators (Hughes et al., 1954). The study in Madras of Devadatta et al. (1960) confirmed these conclusions. Peripheral neuritis can be prevented by the administration of pyridoxine simultaneously with isoniazid (Carlson et al., 1956). Isoniazid administration causes neuritis because of pyridoxine deficiency, which develops due to inactivation of pyridoxine and removal of the coenzyme from tissues through chemical interaction of isoniazid with pyridoxine. Furthermore, isoniazid may compete with pyridoxal phosphate for the enzyme apotryptophanase (Ross, 1958; Robson and Sullivan, 1963).

Thus genetically controlled differences in rates of isoniazid acetylation do carry the clinical implication that slow inactivators tend to develop polyneuritis on long-term therapy in much higher frequency than rapid inactivators. Rapid acetylation of isoniazid is transmitted as an autosomal dominant trait, whereas slow acetylation is inherited as an autosomal recessive trait. In the plasma of rapid inactivators the half-life of isoniazid ranges from 45 to 80 minutes, whereas in slow inactivators the half-life extends from 140 to 200 minutes (Kalow, 1962). Rapid acetylators may excrete unchanged only 3% of a dose, whereas slow acetylators may excrete 30% (Hughes et al., 1954; Peters, 1959, 1960a, 1960b); these differences between rapid and slow inactivators are unrelated to intestinal absorption, protein binding, renal glomerular clearance, or renal tubu-

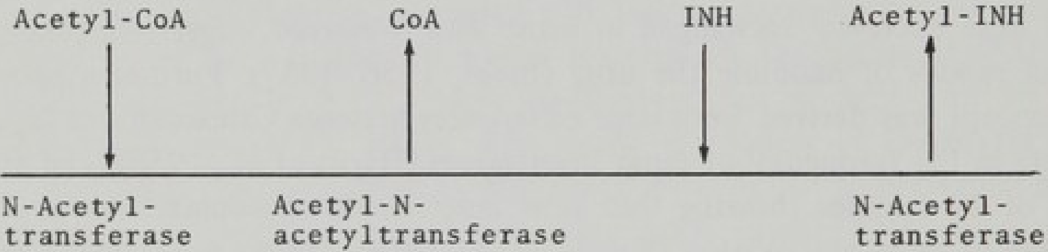
lar reabsorption (Jenne et al., 1961). Their basis lies in the fact that slow inactivators have reduced activity of acetyl transferase (Price Evans and White, 1964; Peters et al., 1965a, 1965b), the liver supernatant enzyme mainly responsible for the metabolism of isoniazid, as well as such other monosubstituted hydrazines as phenelzine, hydralazine, and sulfamethazine (Price Evans, 1965). Acetylation of such other drugs as *p*-aminosalicylic acid and sulfanilamide is monomorphic and probably accomplished by an acetylase different from that which acetylates isoniazid.

A comparison between rates of sulfamethazine and sulfamethoxypyridazine acetylation revealed that the latter drug was acetylated much less than the former (White and Price Evans, 1968). White and Price Evans (1968) reported that rapid inactivators acetylated a greater percentage of sulfamethoxypyridazine than did slow inactivators. It was surprising, however, that serum concentrations of free sulfamethoxypyridazine did not differ significantly in individuals possessing different acetylator phenotype, presumably because of the operation of several other more important factors affecting elimination of the drug from plasma.

Various genetic investigations of isoniazid acetylation have been reported. A twin study revealed the content of free isoniazid in 24-hour samples of urine to be remarkably similar to identical twins, but different in fraternal twins (Bönicke and Lisboa, 1957). Bimodal distributions in the percentage of an isoniazid dose excreted unchanged in urine were observed, suggesting genetically distinct modes of handling the drug (Biehl, 1956, 1957). Further support for this concept was derived from large differences between Caucasian and Japanese subjects in the frequency of rapid inactivators (Harris et al., 1958), and from a study of 20 families showing that slow inactivation of isoniazid was probably recessive to rapid inactivation (Knight et al., 1959). Plasma isoniazid concentrations 6 hours after an oral dose of 9.7 mg/kg in 267 members of 53 Caucasian families were bimodally distributed so that individuals could be categorized as either rapid or slow inactivators (Price Evans et al., 1960). Six hours after the oral dose the mean concentration of isoniazid in plasma was lower in heterozygotes than in rapid inactivators homozygous for the dominant gene; these observations established a dosage effect for the trait (Price Evans et al., 1960). A sensitive microbiologic assay (Sunahara, 1961) permits direct determination of the acetylation genotype. Widely different geographical and racial distributions of the gene have been reported. Of lowest frequency in Eskimos, deficiency is only slightly more common in Far Eastern populations (Motulsky, 1964). In Negroes and European populations, where 70 to 80% of the individuals possess the aberrant gene either in homozygous or heterozygous state, deficiency is common (Armstrong and Peart, 1960; Harris, 1961a; Sunahara, 1961; Price Evans, 1962; Mitchell et al., 1960; Devadatta et al., 1960; Gangadharam and Selkon, 1961; Schmiedel, 1961; Szeinberg et al., 1963; Motulsky, 1964).

Polymorphisms such as the rapid and slow acetylation of isoniazid may be perpetuated by natural selection, such that the gene for slow inactivation possesses advantages in certain environments. Understanding of such hypothetical advantages with respect to isoniazid acetylation will have to await better understanding of the action of the enzyme in vivo. Little information on naturally occurring hydrazine compounds is available, but Peters et al. (1965b) measured dimethylaminobenzaldehyde-reacting substances in urine of individuals not receiving drugs and reported below 0.8 mg total hydrazine equivalents per 12 hours. This value is so low that it may be inferred that the body does not encounter many naturally occurring hydrazines. Neither hexosamine nor tryptophan metabolites appear to be natural substrates for isoniazid acetylase (White and Price Evans, 1967a, 1967b).

The acetylase that transfers an acetyl group from acetyl coenzyme A to isoniazid has been studied in vitro by Price Evans (1962), Price Evans and White (1964), Jenne (1965), and Weber et al. (1968). The latter group purified the enzyme 300- to 500-fold from 100,000-g liver homogenate supernatant which contains all the acetyltransferase activity. Weber et al. (1968) then established for the enzyme a ping-pong mechanism of action. The work of Weber et al. (1968) suggests that the mechanism of INH acetylation may be pictured as follows:



The concept that INH acetylation involves a two-step process of the production and decay of an acetylated enzyme is conveyed in the following partial reactions:

$$N\text{-acetyltransferase} + \text{acetyl-CoA} \rightleftharpoons \text{Acetyl } N\text{-acetyltransferase} + \text{CoA}$$

$$\text{Acetyl-}N\text{-acetyltransferase} + \text{INH} \rightleftharpoons \text{Acetyl–INH} + N\text{-acetyltransferase}$$

Studies of the purified acetylase from rapid and slow INH inactivators revealed that, although the former exhibit approximately twice the amount of enzyme present in the latter, the enzymes isolated from the two acetylator phenotypes are similar with respect to pH optima, heat stability, kinetic behavior, and substrate specifically (Jenne, 1965; Weber et al., 1968; Weber, 1971). These observations might suggest that in man and in rabbit the rapid and slow INH inactivators differ only with respect to the amount of acetylase present in the liver and that qualitatively the enzymes are similar. However, comparison of the electro-

phoretic properties of the rabbit enzyme revealed a possible basis for a structural gene defect. On electrophoretic separation, acetylase activity exhibits two distinguishable bands in both rapid and slow INH inactivators. One of the two components migrates more rapidly toward the anode in the slow than in the rapid acetylator, whereas the slower-migrating component has similar electrophoretic mobility in both phenotypes (LaDu, 1972).

Since phenelzine is polymorphically acetylated, its side effects were observed in rapid and slow acetylators, and severe side effects, including blurred vision and psychosis, were confined mainly to slow acetylators. Reduced incidence of toxicity from a polymorphically acetylated drug in rapid as compared to slow acetylators may explain why toxic effects of the hydrazine drug phtivazid occurred infrequently in those patients who excreted the acetylated form in high concentrations (Smirnov and Kozulitzina, 1962).

Peripheral neuropathy and a syndrome resembling systemic lupus erythematosus occur much more frequently in slow than in rapid acetylators (Perry et al., 1967). Of 57 hypertensive patients receiving 200 to 3000 g of hydralazine for 1 to 15 years, 12 developed symptoms of hydralazine toxicity and all were slow acetylators (Perry et al., 1970). Furthermore, in 153 tuberculous patients on isoniazid, antinuclear antibodies to whole nuclei, nucleoprotein, soluble nucleoprotein, and isoniazid-altered soluble nucleoprotein were detected by complement fixation tests more frequently in slow than in fast acetylators, but this difference was not statistically significant (Alarcón-Segovia et al., 1971). Finally, a relationship between autoimmune reactions to hydralazine and methyldopa has been postulated (Perry et al., 1971). It is claimed that "Caucasian, slow acetylators also have an increased susceptibility to develop IgG antiglobulin reactions following exposure to methyldopa" (Perry et al., 1971).

Suxamethonium Sensitivity or Atypical Pseudocholinesterase

In 1952 shortly after the introduction and widespread use of the muscle relaxant suxamethonium, certain rare patients were found to be abnormally sensitive to it, and several deaths associated with its use were reported (Bourne et al., 1952; Evans et al., 1952). Also called succinylcholine, Suxethonium, Scoline, and Anectine, this drug was initially described in 1906 by Hunt and Taveau, but not until 43 years later were its properties as a muscle relaxant discovered by Bovet et al. (1949). Most commonly employed during general anesthesia, suxamethonium is also used in electroconvulsive therapy and in treatment of tetanus. Short duration of action is its principal therapeutic advantage; the usual dosage of 30 to 100 mg produces muscle paralysis and apnea for approximately 2 min. In atypical patients, the duration of action is 2 to 3 hours. The short duration of action in normal cases is attributable to the exceedingly rapid hydrolysis of suxamethonium by plasma pseudocholinesterase (Bovet-Nitti, 1949). This enzyme removes the choline radicals one at a time, with

formation of the relatively inactive intermediate succinylmonocholine (Lehmann and Silk, 1953; Whittaker and Wijesundera, 1952). Prolonged apnea in patients with atypical pseudocholinesterase can be effectively treated by transfusion of normal plasma or of a highly purified preparation of the human enzyme (Goedde et al., 1968).

In 1952 when the initial reports of bizarre reactions were published, reduced serum pseudocholinesterase activity was noted. With the accumulation of increasing numbers of these rare cases it became clear that such abnormal individuals were otherwise healthy and therefore had low pseudocholinesterase not because of liver disease, poisoning by organophosphorus compounds, malnutrition, or severe anemia, all of which can diminish plasma pseudocholinesterase activity (Lehmann and Ryan, 1956), but rather because of an inherited defect (Forbat et al., 1953). Lehmann and Ryan (1956), on the basis of an investigation of the families of 5 unrelated suxamethonium-sensitive probands, suggested that the disorder was inherited as an autosomal recessive trait. At that time, however, considerable overlap prevented identification of the three phenotypes simply by measurement of plasma pseudocholinesterase activity.

Elucidation of the nature of the enzymatic abnormality was achieved by Kalow and his associates, who demonstrated that it resulted not just from decreased amounts of the normal pseudocholinesterase but rather from the presence of a structurally altered enzyme with kinetic properties markedly different from those of the usual eznyme (Kalow and Genest, 1957; Kalow and Staron, 1957; Kalow and Davies, 1959; Davies et al., 1960). Suxamethonium and other substrates exhibit much lower avidity for the abnormal than for the normal enzyme. At concentrations of suxamethonium present during anesthesia, the abnormal enzyme exerts no detectable effect on the drug, in comparison to the marked hydrolytic activity of the normal enzyme (Davies et al., 1960). The atypical enzyme was more resistant than the normal enzyme to many pseudocholinesterase inhibitors (Kalow and Davies, 1959). At first, differential inhibitors of the normal and atypical enzyme were thought to require a positively charged nitrogen molecule, but fluoride (Harris and Whittaker, 1961) and organophosphorus compounds were each later shown to inhibit the normal and atypical enzyme differentially. Because of the importance of a positive charge on many inhibitors, it was suggested that the positively charged portion of the inhibitor combined with the anionic site of the enzyme; only the anionic site on the atypical enzyme was considered to be defective in either accessibility or magnitude of charge (Kalow and Davies, 1959). Previously two sites had been described on the cholinesterase molecules: an anionic site which accommodated the positively charged choline radical of the substrate and an esteratic site on which the acid portion of the substrate was positioned during hydrolysis (Wilson, 1954). Certain observations of Clark et al. (1968) that the p*K* of the atypical enzyme is lower than that of the usual enzyme, that choline alters the

p*K* of the usual but not the atypical enzyme, and that choline has a lower affinity for the atypical enzyme support the conclusion that the anionic site of the atypical enzyme is altered. However, stimulation by choline of the dephosphorylation step of the usual enzyme, but not of the atypical enzyme, and differential rates of dephosphorylation by sodium fluoride imply that the esteratic site may also be altered in the atypical enzyme (Clark et al., 1968). Two distinct point mutations on the atypical pseudocholinesterase need not be postulated in order to accommodate these data, since alteration of a single residue in the structure of the atypical pseudocholinesterase could modify both the anionic and esteratic sites.

Such detailed studies of the aberrant enzyme permitted development of tests to distinguish the three phenotypes which could not be satisfactorily separated simply by measuring plasma pseudocholinesterase activity. To separate the three phenotypes Kalow and Genest (1957) utilized dibucaine (cinchocaine), a differential inhibitor of normal and atypical pseudocholinesterase. The percentage inhibition of pseudocholinesterase activity produced by 10^{-5} *M* dibucaine was designated the "dibucaine number" or DN. The normal enzyme is inhibited approximately 80%, whereas atypical pseudocholinesterase is inhibited only 20%, and heterozygotes exhibit between 52 and 69% inhibition (Kalow and Genest, 1957). The degree of inhibition is independent of enzyme concentration.

The use of sodium fluoride as an inhibitor led to the discovery of additional genetic variants (Harris and Whittaker, 1962a). Unlike other previously studied compounds, tetracaine is hydrolyzed faster by atypical than by normal pseudocholinesterase, and an even greater separation of phenotypes apparently can be achieved with the procaine-tetracaine ratio than with the DN (Foldes, 1968).

Inheritance of various types of atypical pseudocholinesterase has been suggested by family studies to occur through allelic codominant genes at a single locus (Kalow and Staron, 1957; Harris et al., 1960; Bush, 1961). Four alleles have been identified with the resulting ten genotypes: $E_1{}^uE_1{}^u$, $E_1{}^uE_1{}^a$, $E_1{}^aE_1{}^a$, $E_1{}^sE_1{}^u$, $E_1{}^sE_1{}^s$, $E_1{}^sE_1{}^a$, $E_1{}^fE_1{}^u$, $E_1{}^fE_1{}^f$, $E_1{}^fE_1{}^a$, $E_1{}^fE_1{}^s$ where E_1 signifies the pseudocholinesterase genetic locus and u, a, s, and f indicate the "usual," "atypical," "silent," and "fluoride" sensitive alleles, respectively. Penetrance is complete, although the genes apparently vary in expression (Lehmann and Liddell, 1964). The frequency of homozygous affected individuals was estimated to be 0.019 to 0.017, or approximately 1 in 2800, and approximately 3.8% of individuals in various populations are heterozygotes (Kalow and Gunn, 1959; Kattamis et al., 1962). Individuals who are homozygous recessive for the atypical allele are not as rare as the initial estimates of 1 in 4000 suggested; more intensive population studies increased the number of affected individuals to 1 in 2500 (LaDu, 1972). These revised frequencies suggest the desirability of screening all individuals prior to receiving succinylcholine for their capacity to metab-

olize the drug. Kalow (1965) reported that approximately half the patients investigated because of unusual sensitivity to succinylcholine had a normal activity and type of pseudocholinesterase; the abnormal response to succinylcholine in these subjects may be attributable to inhibition of the enzyme by other drugs.

In a series of 4 families discussed by Lehmann and Liddell (1964) the dibucaine values do not conform to the pattern of autosomal inheritance. These individuals are presumably heterozygous for a rare, so-called silent gene. A few, exceedingly rare individuals with complete absence of serum and liver pseudocholinesterase activity exist (Hodgkin et al., 1965). In these otherwise apparently normal individuals all four normally occurring isozymes of serum pseudocholinesterase were absent; immunodiffusion and immunoelectrophoretic studies indicated the lack of antigenically cross-reacting material (Hodgkin et al., 1965). Heterozygotes for the silent gene exhibit serum cholinesterase activity approximately two-thirds of normal; they overlap considerably with normal values (Hodgkin et al., 1965; Harris et al., 1963). Motulsky (1964) states that such silent mutations may affect the controlling element of the gene, thereby causing complete failure of protein production; he also acknowledges the possibility of a single structural mutation affecting both the active site and the antigenic determinants. Of 5 individuals who were homozygous recessive for the silent gene, Goedde and Altland (1968) reported residual enzymatic activity and antigenic determinants in 3 subjects, each of whom revealed a single band on starch gel electrophoresis at the C-4 position when their sera were concentrated sixfold. Results similar to those of Hodgkin et al. (1965) were obtained in the remaining 2 cases.

Unlike the polymorphism affecting isoniazid acetylation, the incidence of atypical pseudocholinesterase is remarkably unchanged in different geographical areas; however, Gutsche et al. (1967) reported an unusually high incidence of the silent mutation in a population of southern Eskimos. As a result of apnea in 2 Eskimo children after a single low dose of succinylcholine, 19 cases in 11 Eskimo families were ascertained. Only 10 individuals homozygous for the silent gene had been described prior to this survey in Alaska (Szeinberg et al., 1966). The gene frequency of 0.12 in this locality, which extended from Hooper Bay to Unalakleet and centered on the lower Yukon River, led to an estimation that 1.5% of this Alaskan population was sensitive to succinylcholine. The authors suggested that the high frequency of the rare silent gene occurred in this, but not other, regions of Alaska because of the isolation and consequent inbreeding of the population. However, only 2 of the 11 affected Eskimo families are known to be related. Alternatively, the gene may have been favored by certain characteristics of the environment. Of 17 affected Eskimos, 8 deficient persons had pseudocholinesterase activities of two to eight units, according to a method adapted to permit analysis of greater volumes of sera, whereas 9 individuals exhibited no activity whatever. Possibly trace pseudocholinesterase activity represents a different mutation from that characterized by no detectable activity.

Close resemblance of most populations in their gene frequencies of atypical pseudocholinesterase may indicate either that little selective advantage is conferred now by the various genotypes or that the pertinent environmental conditions are similar in widely different countries. Such solinaceous plants as tomatoes and potatoes possess a potent differential cholinesterase inhibitor (Orgell et al., 1958) shown by Harris and Whittaker (1962b) to be the glycoalkaloid solanine. Since atypical pseudocholinesterase is less sensitive to inhibition by this naturally occurring substance than is the normal enzyme, it has been suggested that in cases of solanine poisoning the atypical genotype would be at a selective advantage, and, before the pseudocholinesterase phenotypes could be readily identified, several outbreaks of solanine poisoning were reported (Wilson, 1959; Willimott, 1933; Harris and Cockburn, 1918).

Plasma pseudocholinesterase activities may be elevated in several disease states such as thyrotoxicosis, schizophrenia, hypertension, and acute emotional disorders, and after concussion; increases also occur as a genetically transmitted condition without overt clinical manifestations, but associated with an electrophoretically slower migrating C_4 isozyme (Harris et al., 1963; Neitlich, 1966). In 1029 male military personnel between ages 17 and 35, an individual was discovered whose plasma pseudocholinesterase activity of 1278 units was more than three times higher than the mean for all the volunteers (Neitlich, 1966). A family study revealed that the sister and daughter of the propositus had values of 1518 and 1237 plasma pseudocholinesterase units, respectively, and that his mother had 566 units. This variant, termed the Cynthiana variant, was reinvestigated by Yoshida and Motulsky (1969); and, as expected, the unusually high pseudocholinesterase activity was associated with resistance to the pharmacological effects of succinylcholine. Because the physiochemical and kinetic properties of the enzyme are normal, the Cynthiana variant may result from a defect of a regulator gene controlling pseudocholinesterase activity rather than from a structural gene abnormality. Previously Kalow and Genest (1957) and Kalow and Staron (1957) described an individual with 2.5 times the average pseudocholinesterase activity of 1556 subjects, but a family study was not performed. Approximately 10% of a random sample of the British population had slightly higher-than-normal pseudocholinesterase activity associated with a retarded electrophoretic mobility of the main isozyme (Harris et al., 1963). This slower moving band was designated C_5. Neitlich's pseudocholinesterase variant also exhibited slower electrophoretic mobility than the normal C_4 isozyme; however, the greatly elevated total plasma pseudocholinesterase activity of the American variants distinguished them from the variants described in England. As might have been anticipated, individuals possessing markedly elevated plasma pseudocholinesterase activity are resistant to the usual doses of suxamethonium.

Recent studies on the isolation of the multiple electrophoretic forms of serum pseudocholinesterase suggest that, although differing in molecular weight, they are interconvertible and exhibit similar kinetic behavior (LaDu, 1972). The

electrophoretic properties of the atypical pseudocholinesterase resemble those of the normal enzyme (LaDu, 1972).

Deficient Parahydroxylation of Diphenylhydantoin

It is well known that many lipid-soluble drugs are rendered more water-soluble and hence more excretable through metabolism by enzyme systems in liver microsomes (Gillette, 1963, 1966). Most of these liver microsomal enzymes are oxidases requiring oxygen, NADPH, and cytochrome P-450; their characterization remains to be accomplished because these enzymes are exceedingly sensitive, losing most of their activity when removed from the endoplasmic reticulum. Deficient parahydroxylation of Dilantin (diphenylhydantoin) is the earliest published example of a genetic defect of mixed function oxidases in humans (Kutt et al., 1964b). In man Dilantin is metabolized mainly by parahydroxylation of one of the phenyl groups to yield 5-phenyl-5′-parahydroxyphenylhydantoin, HPPH, which is conjugated with glucuronic acid and then eliminated in urine (Butler, 1957; Woodbury and Esplin, 1959; Maynert, 1960).

One of the most commonly used anticonvulsants since its introduction by Merritt and Putnam in 1938, Dilantin causes multiple toxic reactions including nystagmus, ataxia, dysarthria, and drowsiness. Yahr et al. (1952) stated that 77% of patients develop toxicity on a daily dose of 0.6 g which is not above the amount recommended by these and other authors (Yahr and Merritt, 1956). These toxic reactions have been clearly shown by Kutt et al. (1964a) to be dose related (Fig. 3). In the propositus W. J., toxic symptoms developed on a commonly used dosage of 4.0 mg/kg, but not on a dose of 1.4 mg/kg. Prolonged high blood levels of unchanged Dilantin occurred in combination with abnormally low urine levels of the metabolite HPPH (Kutt et al., 1964b). A study of the family of W. J. (Fig. 6) revealed 2 affected and 3 unaffected individuals in two generations, suggesting that low activity of Dilantin hydroxylase exhibits dominant transmission.

The patient's capacity to parahydroxylate other compounds, such as phenobarbital and phenylalanine, was normal; apparently, therefore, parahydroxylation of these drugs is accomplished by enzymes different from those that hydroxylate Dilantin. However, since urinary excretion of unaltered phenobarbital (Butler, 1956) occurs in higher amounts, reaching 30% of the daily intake, than does Dilantin, which attains only 5% of daily intake, Kutt et al. (1964b) suggested that phenobarbital accumulation is less likely to occur than Dilantin accumulation. Therefore, a hydroxylation defect of phenobarbital may be masked.

When toxic symptoms develop in patients receiving Dilantin, particularly when low dosages are administered, determinations of blood concentrations and urinary metabolites of Dilantin should be performed. If the patient is particularly sensitive to Dilantin and deficient in hydroxylating capacity, discontinua-

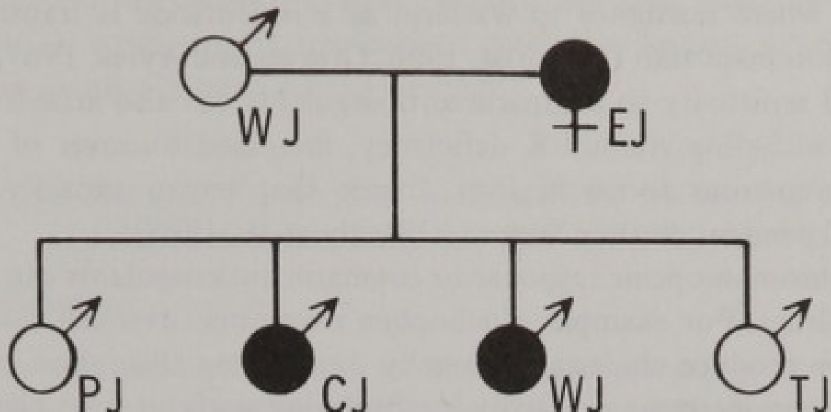


FIG. 6.–Pedigree of deficient parahydroxylation of diphenylhydantoin with propositus W. J. and his affected brother, C. J., and mother, E. J., reproduced from Kutt et al. (1964b).

tion of the drug is not necessary. Rather adjustment of its dosage should be made to give the desired blood concentrations.

A much more prevalent cause of Dilantin intoxication than heritable deficiency of parahydroxylase activity has recently been identified as slow inactivation of isoniazid (Brennan et al., 1968). All 5 patients who developed clinically evident Dilantin toxicity in a series of 29 individuals receiving Dilantin (300 mg daily for 3 weeks) were very slow isoniazid inactivators. In rat liver microsomes both isoniazid and *p*-aminosalicylic acid interfered with Dilantin parahydroxylation (Kutt et al., 1968).

Bishydroxycoumarin Sensitivity

Solomon (1968) reported bishydroxycoumarin sensitivity in a patient who received the drug for treatment of an acute myocardial infarction. The patient's plasma bishydroxycoumarin half-life of 82 hours on a dose of 150 mg compared to normal values of 27 ± 5 hours. Although family studies were not performed because of unwillingness to cooperate, the patient's mother suffered a spinal cord hematoma, causing permanent paraplegia, while she was receiving a small weekly dose of 2.5 to 5 mg of warfarin. This unfortunate event suggests not only the possibility of hereditary transmission of bishydroxycoumarin sensitivity, but also the desirability of measuring drug concentrations in blood during long-term therapy.

Warfarin and bishydroxycoumarin are known to be extensively hydroxylated in rats (Ikeda et al., 1966; Christensen, 1966), but in humans the metabolites have not yet been fully characterized. The metabolic defect in this patient with bishydroxycoumarin sensitivity may involve a hepatic microsomal hydroxylase that possibly is deficient in both him and his mother.

In rabbits, genetic factors have been shown to influence responsiveness to anticoagulants (Smith, 1939; Link, 1944; Solomon and Schrogie, 1966), as they

do in rats, where resistance to warfarin as a rodenticide is transmitted as an autosomal dominant trait (Editorial, 1966; Greaves and Ayres, 1967).

Increased sensitivity to coumarin anticoagulants can also arise from acquired conditions, including vitamin K deficiency, increased turnover of plasma proteins, and numerous forms of liver disease that impair capacity to produce vitamin K-dependent clotting factors (O'Reilly et al., 1968).

The prothrombinopenic response to coumarin anticoagulants can be increased by various drugs. For example, cinchophen may cause liver cell damage; phenothiazine may produce cholestasis, thereby diminishing absorption of vitamin K; phenylbutazone increases sensitivity by displacing warfarin from plasma albumin (Aggeler et al., 1967); and phenyramidol inhibits the hepatic microsomal enzymes responsible for metabolism of coumarin drugs (O'Reilly and Aggeler, 1965).

Acetophenetidin-Induced Methemoglobinemia

In 1967 Shahidi reported severe methemoglobinemia and hemolysis in a 17-year-old girl after phenacetin ingestion. Several erythrocyte enzymes, including G-6-PD, 6-phosphogluconate dehydrogenase, diaphorase, and glutathione reductase, and the concentration of reduced glutathione were normal. No physiochemical abnormalities of hemoglobin were detected, so that the factor or factors causing hemolysis and as much as half of the patient's hemoglobin to be on certain occasions in the form of methemoglobin seemed to be extracorpuscular compounds. The explanation for the effects of phenacetin (acetophenetidin) on this patient was discovered in her urine, which after drug administration contained large amounts of 2-hydroxyphenetidin and 2-hydroxyphenacetin derivatives. In normal individuals phenacetin in a dose of 2 g produced no more than 2.8% methemoglobin, and more than 70% of the dose appeared in the urine as *N*-acetyl-*p*-aminophenol with only small amounts of the hydroxylated products so prominent in the patient's urine. The patient's 38-year-old sister resembled her in exhibiting an abnormal response to phenacetin, but another sister, a brother, and both parents revealed the normal response to phenacetin.

These observations suggested autosomal recessive inheritance of a defect in which the patient's hepatic microsomal mixed-function oxidases were deficient in deethylating capacity. Instead of being deethylated, as in normal individuals, phenacetin was hydroxylated in the patient and her 38-year-old sister. These hydroxylated products of phenacetin identified in their urine were probably responsible for the toxicity observed after phenacetin administration. If this hypothesis was correct, induction of the hepatic microsomal phenacetin hydroxylating enzymes prior to phenacetin administration should worsen the clinical picture by increasing the concentration of toxic hydroxylated phenacetin metabolites. To test the hypothesis, the patient was pretreated with phenobarbital and then given phenacetin. Profound methemoglobinemia ensued and severe neuro-

logical symptoms including bilateral positive Babinski responses developed (Shahidi, 1967). After similar treatment a normal volunteer exhibited neither methemoglobinemia nor neurological changes.

GENETIC CONDITIONS PROBABLY TRANSMITTED AS SINGLE FACTORS ALTERING THE WAY DRUGS ACT ON THE BODY

Warfarin Resistance

Genetically controlled resistance to warfarin occurs rarely in man. In a 71-year-old patient receiving anticoagulants for a myocardial infarction, such resistance was carefully documented and his family studied for the lesion (O'Reilly et al., 1964). Other than a reproducible reduction in his one-stage prothrombin concentration to approximately 60% of normal, the patient exhibited no abnormalities by physical or laboratory examination. Because of the patient's low prothrombin time, anticoagulants were initially withheld. After 1 month they were administered, at which time he proved to be resistant, rather than sensitive, to dicoumarol. A daily dose of 20 mg of warfarin failed to achieve any prothrombinopenic response, and a daily dose of 145 mg was required to reduce the prothrombin concentration to therapeutic levels. In 105 patients on long-term anticoagulant therapy the mean daily dose of warfarin was 6.8 ± 2.8 mg (O'Reilly et al., 1968), so that the resistant patient was 49 standard deviations above the mean.

Five other members of the family in three generations were also resistant to warfarin (Fig. 7), and autosomal dominant transmission of the trait was suggested by the facts that both sexes were equally affected and that representatives from all three generations were resistant to warfarin.

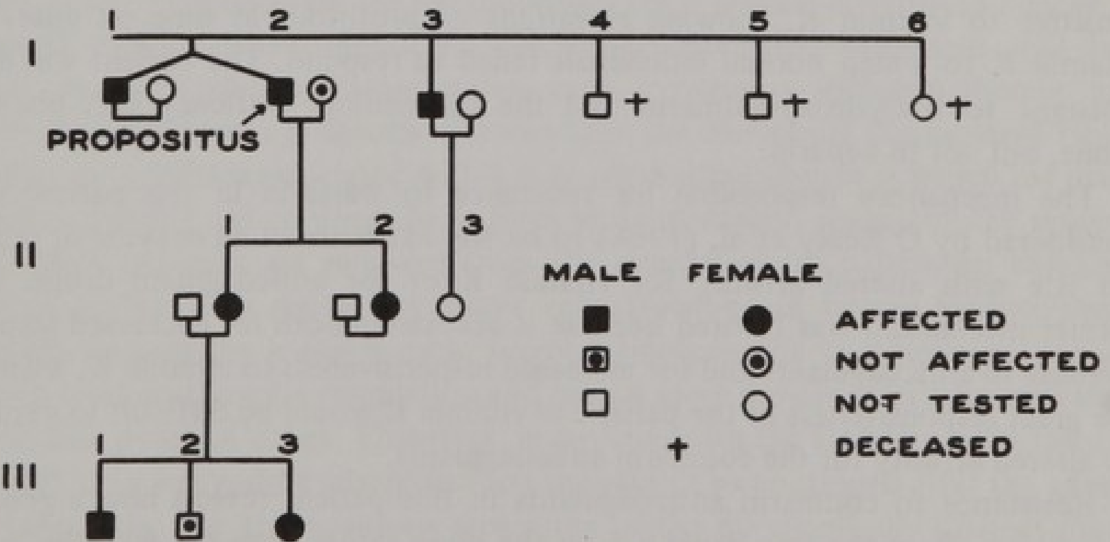


FIG. 7.–Pedigree of family M, indicating the incidence of resistance to coumarin anticoagulant drugs, reproduced from O'Reilly and Aggeler (1965).

Resistance to coumarin anticoagulant drugs ensues from various environmental conditions. Decreased sensitivity to the prothrombinopenic effect of coumarin drugs has been reported in hyperthyroid patients treated with propylthiouracil, in patients with congestive heart failure relieved by operative procedures or medications, and in patients with liver disease being treated medically (Elias, 1965). Large doses of coumarin drugs are required to offset increased levels of vitamin K-dependent clotting factors released during pregnancy (O'Reilly et al., 1968). Simultaneous administration of the natural antidote, vitamin K, and of other therapeutic agents can produce resistance to the coumarin anticoagulants. Barbiturates, glutethimide, chloral hydrate, and griseofulvin are inducing agents that can reduce the blood concentration of anticoagulant drugs by stimulating their metabolism.

To identify the mechanism of the defective response to warfarin, O'Reilly et al. (1964) performed various pharmacodynamic studies on their patient before initiating long-term therapy. After the standard oral dose of 1.5 mg of warfarin sodium per kilogram of body weight, blood concentrations of the anticoagulant were determined serially. The plasma warfarin concentrations rose rapidly after an oral dose, indicating that the drug was absorbed normally from the gastrointestinal tract. The concentration of warfarin attained in plasma and its rate of elimination from plasma were normal, suggesting a normal volume of distribution and a normal rate of metabolism of the drug (O'Reilly et al., 1968). The degree of binding of warfarin to the patient's plasma proteins was identical to that of normal subjects. Electrophoretic studies showed that warfarin was bound exclusively to albumin, as in normal plasma. Warfarin was not excreted unchanged in urine or stools, even after administration of very high doses. A metabolite of warfarin was recovered from the patient's urine in amounts similar to those recovered from the urine of normal subjects given equivalent amounts of drug. While on high doses of warfarin, the patient was shown to be unusually sensitive to vitamin K, showing elevations of prothrombin time on doses of vitamin K to which normal individuals failed to respond. The patient was also resistant to bishydroxycoumarin and the indanedione anticoagulant phenindione, but not to heparin.

The mechanism responsible for resistance to warfarin in this patient was considered by O'Reilly et al. (1968) to be the existence of an enzyme or receptor site with altered affinity for vitamin K or for anticoagulant drugs. The former mechanism was favored because it accounted both for decreased responsiveness to anticoagulants and for increased responsiveness to vitamin K, whereas the great responsiveness of the patient to vitamin K would be difficult to explain by altered affinity for the coumarin anticoagulants.

Resistance to coumarin anticoagulants in this patient reveals how a genetic defect can affect the way drugs act on the body rather than the way the body transforms or metabolizes drugs. Coumarin anticoagulants are antimetabolites

that function by competing with the natural substrate vitamin K for receptor sites in an enzyme system responsible for the synthesis of clotting factors II, VII, IX, and X (O'Reilly et al., 1968). If the receptor site is so altered that its avidity for vitamin K is increased, much higher concentrations of anticoagulant would be required to compete effectively with vitamin K, thereby reducing synthesis of the clotting factors and decreasing prothrombin times.

In 1970 O'Reilly reported a second large kindred involving 18 cases of warfarin resistance in two generations. The pedigree was consistent with autosomal dominant transmission of the trait. The metabolism of sodium warfarin was normal, but the affected individuals were exquisitely sensitive to vitamin K. The data from this kindred substantiated the interpretation based on the earlier study that warfarin resistance resulted from a mutation of the receptor site for vitamin K and oral anticoagulants (O'Reilly, 1970).

G-6-PD Deficiency, Primaquine Sensitivity, or Favism

The most common hereditary enzymatic abnormality in man, G-6-PD deficiency, is transmitted as an X-linked incomplete dominant. G-6-PD deficiency is actually a series of disorders; more than 80 physiochemically discrete variants of the molecule exist, each variant being associated with a slightly different clinical picture (Motulsky et al., 1971). Thus, G-6-PD deficiency illustrates the important principle of genetic heterogeneity of conditions initially considered homogeneous encountered on a smaller scale in some of the pharmacogenetic disorders reviewed above. The initial discovery that a certain structural alteration in an enzyme produces a certain clinical syndrome is frequently followed by observations of new, chemically distinct mutations affecting the same protein. The clinical signs and symptoms of the new mutation may differ considerably from those accompanying the old mutation. These clinical differences may be so marked, as with certain mutations affecting the hemoglobin molecule, that they form clearly separable disease states, each requiring a different therapy and possessing a different prognosis. For example, the commonly encountered Negro variant of G-6-PD is associated with a mild self-limited anemia in which the drugs listed in Table 1 can continue to be given without danger, since only the susceptible older red cells are removed from the circulation by hemolysis; these are rapidly replaced by resistant younger cells. However, in various Mediterranean G-6-PD variants the lesion is more severe; hemolysis affects a larger proportion of the total erythrocyte population and occurs more rapidly after administration of smaller doses of drugs. Therefore, in patients with the Mediterranean form of G-6-PD deficiency, drug administration should not be continued after the advent of hemolysis. For these reasons separation of the 80 chemically distinct mutations affecting the G-6-PD molecule according to the severity of clinical symptoms is useful. Motulsky et al. (1971) have associated the type of G-6-PD

variant with the severity of enzyme deficiency. Variants have been characterized by several properties including the total G-6-PD activity in erythrocytes, electrophoretic mobility of the G-6-PD, K_m for NADP and G-6-P, rate of utilization of substrate analogs, and heat stability. Furthermore, microfingerprinting techniques permitted definition of the specific amino acid substitution which has been determined in G-6-PD A^+ (Yoshida, 1967) and in G-6-PD Hektoen (Yoshida, 1970). Motulsky et al. (1971) have related the severity of hemolysis to the amount of G-6-PD activity in erythrocytes. Only erythrocytes older than 50 days are hemolyzed in the common Negro type of deficiency, whereas in the Mediterranean type a larger fraction of the erythrocytes is destroyed due to the fact that in Mediterranean variants the mutation is expressed by severe reduction of G-6-PD activity in relatively young cells. In the Negro variant young erythrocytes have sufficient G-6-PD activity to withstand the oxidant effect of drugs. There is a need to establish a relationship of G-6-PD activity to erythrocyte age in most of the 80 distinct mutations of the enzyme.

Several reviews of G-6-PD deficiency have been published (Motulsky, 1964; Vesell, 1969; Motulsky et al., 1971; Motulsky, 1972) so that the many contributions to our understanding of this important pharmacogenetic disorder need not be restated here. Particular attention has previously been given to G-6-PD deficiency as a prototype X-linked condition confirming the Lyon hypothesis and as a balanced polymorphism conferring a selective advantage in certain environments by way of protection against severe falciparum malaria.

In spite of numerous genetic studies on different G-6-PD variants, several important issues concerning this disorder remain unresolved. These include (1) the specific biochemical mechanisms by which drug administration causes hemolysis and (2) development of a satisfactory in vitro test system to determine the potential of new drugs for producing hemolysis in subjects possessing different G-6-PD variants. Despite many studies on this first issue, the events leading to drug-induced hemolytic anemia remain somewhat obscure. The drugs themselves are metabolized in a normal fashion by the body, but they or more likely their hydroxylated metabolites cause damage because of increased fragility of G-6-PD-deficient erythrocytes. Normally, erythrocytes withstand oxidative compounds and maintain their glutathione in a reduced state through glutathione reductase, which by means of NADPH regenerates GSH from oxidized glutathione (GSSG). NADPH is formed by both G-6-PD and 6-phosphogluconate dehydrogenase (6-PGD), the first two enzymes in the hexose monophosphate shunt or phosphogluconate oxidative pathway. This oxidative route provides only a small amount (approximately 10%) of the total metabolic energy of the erythrocyte, the major part being accounted for by the Embden-Myerhof pathway. However, the relative rates of glycolysis are appreciably altered by such factors as the pH of the suspending medium, the G-6-PD activity, and the rate of TPNH oxidation. Deficiency of G-6-PD activity reduces the amount of TPNH

available to the erythrocyte and thereby the concentration of GSH. GSH is apparently necessary in maintaining –SH groups on critical proteins of the erythrocytes in a reduced state (Barron and Singer, 1943), and GSH deficiency with normal G-6-PD activity causes a nonspherocytic, congenital hemolytic anemia with drug sensitivity (Oort et al., 1961; Prins et al., 1963). This would seem to indicate the importance of GSH in the metabolic economy of the erythrocytes; on the other hand, red cell survival is not decreased by complexing most of the GSH in erythrocytes with *N*-ethylmaleimide (Jacob and Jandl, 1962).

An investigation of the effect on mechanical fragility of exposing normal and G-6-PD-deficient erythrocytes to various compounds and their metabolites suggested the following sequence of events in drug-induced hemolysis (Fraser and Vesell, 1968; Fraser et al., 1971a, 1971b): the drug is first metabolized to a product more susceptible to further oxidation. This metabolite is converted to an oxidant intermediate by the erythrocyte. The oxidant intermediate then causes damage to the erythrocyte membrane, particularly in old cells possibly by oxidation of reduced sulfhydryl groups (Weed and Reed, 1966). We regard mechanical fragility as an index of this membrane damage. Fragmentation of old erythrocytes ("hemolysis") in the circulation probably results from this membrane damage, although splenic sequestration probably also participates as an additional mechanism (Weed and Reed, 1966).

The metabolism of the erythrocyte is unusual in that it must function without the benefit of a nucleus. The red cell apparently is unable to synthesize protein but does synthesize certain simpler substances such as GSH, DPN, and ATP. It requires energy sources for maintaining concentration gradients of sodium and potassium and for continual reduction of methemoglobin. The glycolytic and oxidative pathways of glucose metabolism provide this energy source. But as the normal cell ages certain enzymes including G-6-PD lose activity (Marks et al., 1958; Marks and Gross, 1959). In G-6-PD-deficient cells, G-6-PD activity declines with age at a faster than normal rate (Marks and Gross, 1959). Clearly, therefore, older cells of individuals possessing mutations of their G-6-PD are more susceptible to lysis than younger cells.

Clinical studies of individuals bearing various G-6-PD mutations corroborate the view that older cells are more vulnerable to the hemolytic action of drugs than are younger ones. A mild form of disorder is observed in Negroes. When a Negro subject receives 30 mg of primaquine daily, no hemolysis occurs for 2 or 3 days, but the urine may turn black. Further signs may not develop, but in more severe cases there may be weakness, abdominal and back pain, icterus, and black urine. Heinz (inclusion) bodies may appear in the erythrocytes (Beutler et al., 1954). Anemia and reticulocytosis supervene. In approximately 1 week this "acute hemolytic phase" ends spontaneously even in the face of continued drug administration, and the "recovery phase" begins (Beutler, 1966). The patient

feels improved, and the abnormalities noted above regress, even though administration of drug is continued. The Coombs' test is negative; and the osmotic fragility is normal, although the mechanical fragility is increased (Fraser and Vesell, 1968). This refractory state has been attributed not to any change in the metabolism of the drug or in the reactivity of the erythrocytes, as in immunological phenomena, but rather to a change in composition of the erythrocyte population. The older, more sensitive cells with their greater relative deficiency have been eliminated. The younger remaining cells with higher G-6-PD activities resist the osmotic and oxidant effects of various drugs and their metabolites (Dern et al., 1954b). Subjects with Mediterranean G-6-PD deficiency exhibit a similar, but more severe, clinical course. In some studies the anemia is not self-limited (Salvidio et al., 1963); in others it is (Larizza et al., 1958). In Caucasians the spectrum of drugs causing hemolysis includes fava beans and chloramphenicol and is wider than in Negro subjects (Beutler, 1966).

The need for determining in vitro the hemolytic potential of new drugs remains. Several such tests have been suggested. These include measurement of the mechanical fragility of normal and G-6-PD-deficient erythrocytes (Fraser and Vesell, 1968; Fraser et al., 1971a, 1971b), and measurement of $^{14}CO_2$ evolution from glucose-1-^{14}C in erythrocytes removed from normal volunteers both before and after primaquine ingestion (Welt et al., 1971). Primaquine ingestion significantly increased $^{14}CO_2$ evolution. Even though the degree of stimulation was variable and small, this technique might eventually prove useful and should be investigated in G-6-PD-deficient individuals. Any test for hemolytic potential in vivo based entirely on procedures in vitro fails to reflect the complexity of in vivo mechanisms that depend also on such events as splenic sequestration. A somewhat dangerous test, which is technically sound although obviously not suitable for the routine screening of new drugs, involves measurement of changes in the survival of G-6-PD-deficient radioactively labeled cells transfused into G-6-PD-normal subjects before and after administration of the new drug.

In certain G-6-PD variants hemolysis may apparently occur spontaneously or during infection; clearly several environmental alterations in addition to those produced by drug administration can place sufficient stress on the metabolism of G-6-PD-deficient erythrocytes to cause hemolysis. In this connection the role of hepatic microsomal drug-metabolizing enzymes in converting alkaloids present in foods to more occidant compounds capable of damaging erythrocyte membranes should be investigated; possibly in fever the activity of these enzymes is increased and higher concentrations of occidant metabolites might develop (Song et al., 1971).

Drug-Sensitive Hemoglobins

Investigation of a life-threatening hemolytic anemia after administration of sulfa drugs in a 2-year-old girl and her father resulted in the discovery of a new

hemoglobin and a new pharmacogenetic entity (Hitzig et al., 1960; Frick et al., 1962). The girl developed severe hemolysis at age 2 after receiving sulfadimethoxine for a fever of unknown origin. A history was elicited of a milder episode at 7 months of age when sulfonamides were given for an ear infection. Since childhood the father had experienced repeated, but mild, occurrences of jaundice and dark urine, episodes not invariably associated with drug administration. After receiving a sulfonamide for dysuria, he had a severe hemolytic crisis.

The father and daughter possessed an abnormal hemoglobin comprising 20 to 30% of the total pigment with electrophoretic mobility between that of hemoglobins A and S. The β chain was abnormal: arginine substituted for the usual histidine residue at the sixty-third position, which is particularly important because it is here that the heme group is attached to the β chain (Muller and Kingma, 1961; Huisman et al., 1961).

Of 65 relatives examined, 15 exhibited the abnormal hemoglobin, designated hemoglobin Zürich. The defect is transmitted as an autosomal dominant trait (Frick et al., 1962). When red cells from the father were transfused to a normal volunteer, the half-life of the transfused erythrocytes was calculated to be 11 days instead of the normal 120. Administration of either sulfonamides or primaquine caused rapid disappearance of the transfused cells (Frick et al., 1962). In a second family ascertained in Maryland with the same substitution at the sixty-third position of the β chain, the severity of the hemolytic episodes was less than in the Swiss cases (Rieder et al., 1965).

Another drug-sensitive hemoglobin, designated hemoglobin H, is a special form of α-thalassemia. Composed of four β chains (Baglioni, 1963), hemoglobin H is sensitive to the oxidant drugs described under G-6-PD deficiency. In certain regions of the world, such as Thailand, the frequency of hemoglobin H disease, which is transmitted as an autosomal recessive, is high, occurring in 1 of 300 individuals born in Bangkok. The levels of hemoglobin H in affected subjects vary from 5 to 30%. Since hemoglobin H is almost incapable of carrying oxygen at physiological tensions, subjects with a high proportion of this hemoglobin are at great disadvantage. In hemoglobin H disease, α-chain synthesis is considerably slower than β-chain synthesis.

Two drug-sensitive, rare, unstable hemoglobins, probably transmitted as autosomal codominants, are hemoglobins Torino and Shepards Bush. Sulfonamides provoked hemolytic crises in one member of the family possessing hemoglobin Torino (Prato et al., 1970). Hemoglobin Shepards Bush was discovered because of persistent reticulocytosis in a South African woman of British extraction (White et al., 1970). The patient's mother had the same abnormal hemoglobin and experienced severe hemoglobinuria after sulfonamides.

Taste of Phenylthiourea or Phenylthiocarbamide (PTC)

Capacity to taste the compound phenylthiourea, also called phenylthiocarbamide or PTC, is transmitted as an autosomal dominant trait. Tasters are either

heterozygous or homozygous; inability to taste PTC is inherited as an autosomal recessive trait (Snyder, 1932; Blakeslee, 1932; Kalmus and Hubbard, 1960). This polymorphism was discovered in 1932 when Fox, who synthesized the compound, reported that he could not perceive a bitter taste emanating from dust arising when the powder was poured into a container. A colleague working in the same room complained of the bitter taste (Fox, 1932).

Harris and Kalmus (1950a) developed a refined test for PTC tasting and quantitated thresholds by making 14 serial dilutions of PTC with water. Tumblers containing PTC in increasing concentrations were alternated with tumblers of water. Females detect PTC in greater dilutions than males; tasting sensitivity decreases with age; and various other compounds containing the N–C=S grouping also exhibit a bimodality in taste perception so that the N–C=S group in PTC seems to be responsible for such differences (Harris and Kalmus, 1950b).

Ability to taste PTC is related in several ways to thyroid disease. Goiter in the rat can be produced by PTC administration (Richter and Clisby, 1942). The same bimodality in taste perception exhibited by subjects to PTC is also displayed to compounds related to PTC by possessing the N–C=S group, such as the antithyroid drugs methyl and propylthiouracil. Harris et al. (1949) reported that 41% of 134 patients with nodular goiter were nontasters, and this observation was confirmed by Kitchin et al. (1959) in 447 individuals submitted to thyroidectomy for various reasons. A marked increase in nontasting frequency occurred in male patients with multiple thyroid adenomas. In females, cyclic changes of thyroid involution and hyperplasia occurring with the menstrual cycle may be a significant cause of thyroid disease and therefore may conceal the true connection of PTC tasting and thyroid disorders (Kitchen et al., 1959). These authors also observed that in patients of either sex markedly low frequencies of nontasters occur in toxic diffuse goiter. Nontasters are apparently more susceptible to athyreotic cretinism (Fraser, 1961; Shepard and Gartler, 1960) and also to adenomatous goiter. Tasters develop toxic diffuse goiter more frequently than nontasters.

The grouping S–C=N is goitrogenic, and a compound containing it has been isolated from turnip, cabbage, brussels sprouts, kale, and rape (Greer, 1957; Clements and Wishart, 1956). The substance containing this grouping, which is closely related to the N–C=S grouping of PTC, has been identified as 1,5-vinyl-2-thiooxazolidone, a compound generated from an inactive precursor by an enzyme in the plant. Cooking reduces the concentration of the goitrogen; however, during winter, cattle consume several of these plants and may transmit the goitrogen in their milk (Clements and Wishart, 1956).

PTC nontasters occur more frequently in open-angle glaucoma than in the general population, and significantly less frequently in angle-closure glaucoma (Becker and Morton, 1964).

Geographical variations occur in the frequency of nontasters: 31.5% of

Europeans (Saldanha and Becak, 1959), 10.6% of Chinese, and 2.7% of Africans are nontasters (Barnicot, 1950). The reasons for different geographical distributions of the gene for PTC tasting are obscure. With respect to capacity to metabolize methylthiouracil and thiopentone, tasters and nontasters reveal no differences (Price Evans et al., 1962). Other approaches to the enigma should be sought. Possibly related are the observations that patients with adrenal cortical insufficiency exhibit greater sensitivity than normal to taste and to olfaction, sensitivities that can be returned to normal by the administration of carbohydrate-active steroids (Henkin et al., 1963; Henkin and Bartter, 1966).

Responses of Intraocular Pressure to Steroids: Relationship to Glaucoma

A polymorphism exists in the response of ocular pressure of normal subjects to topical steroids (Armaly, 1968). In 80 normal individuals elevations in intraocular pressure after local administration of 0.1% opthalmic solution of dexamethasone 21-phosphate exhibit a trimodal distribution (Armaly, 1968). The steroid was applied to the right eye daily for 4 weeks, ocular pressure was measured weekly, and elevations in pressure after 4 weeks were determined. Table 6 shows the trimodal distribution of individuals in the extent of their increases in intraocular pressure over this 4-week period. A genetic hypothesis,

TABLE 6.–*Genotype Classification of Dexamethasone Hypertension and Frequency Distributions in Different Clinical Categories**

		Percent frequency of genotypes		
Category	No. of subjects tested	Low (p^Lp^L), ΔP < 6 mm Hg	Intermediate (p^Lp^H), ΔP 6-15 mm Hg	High (p^Hp^H), ΔP > 15 mm Hg
Limits of pressure rise (mm Hg)		5 or less	6-15	16 or more
Mean pressure rise (mm Hg)		1.96	10.0	19.5
Standard deviation (mm Hg)		±2.00	±2.5	†
Genotype		p^Lp^L	p^Lp^H	p^Hp^H
Random sample	80	66%	29%	5%
Open-angle hypertensive glaucoma	33	6%	48%	44%
Low-tension glaucoma	15	7%	53%	40%
Normal eye in recessed-angle glaucoma	15	–	53%	47%
Normal eye in angle recession without glaucoma	4	75%	25%	–

* From Armaly (1968).

† Range in sample 18-22 mm Hg.

confirmed by family studies, suggests the existence of three genotypes: $P^L P^L$ for low elevations of 5 mm Hg or less, $P^L P^H$ for intermediate increases from 6 to 15 mm Hg, and $P^H P^H$ for high increment in pressure of 16 or more mm Hg.

An association between certain types of response and glaucoma was indicated (Armaly, 1968). In a sample of open-angle hypertensive glaucoma and also of low-tension glaucoma, the distribution of responses (Table 6) differed from that in the random sample of normal subjects shown in Table 6 (Armaly, 1968). In both conditions and surprisingly in the uninvolved eye of patients with unilateral posttraumatic glaucoma, a marked reduction in $P^L P^L$ genotypes and a corresponding increase in $P^L P^H$ and $P^H P^H$ genotypes occurred (Table 6).

Family studies of individuals with glaucoma suggested that the response of high elevations of intraocular pressure after dexamethasone administration was inherited as an autosomal recessive trait. Armaly concluded that although affliction with glaucoma can occur with genotypes other than $P^H P^H$ and $P^H P^L$, the P^H gene is closely associated with the development of the types of glaucoma listed in Table 6.

In acute angle-closure glaucoma, also called narrow-angle glaucoma, genetic factors have been described which determine chamber angles (Kellerman and Posner, 1955) and chamber depths (Törnquist, 1953). However, these studies revealed environmental factors that could also exert appreciable effects on chamber depths. In individuals who have inherited narrow chambers, dilatation of the pupils can precipitate acute attacks of glaucoma (Grant, 1955). Several mydriatic agents can produce these acute attacks in genetically susceptible individuals. These drugs include various adrenergic compounds such as epinephrine, phenylephrine, ephedrine, and cocaine; in addition, drugs such as atropine and scopolamine that block the effects of cholinergic nerves also may cause an attack of angle-closure glaucoma (Kalow, 1962).

Malignant Hyperthermia with Muscular Rigidity

The hereditary nature of the malignant hyperthermia with muscular rigidity that occurs very rarely during the course of anesthesia with the agents listed in Table 1 was first suggested by the report of Denborough et al. (1962). They described a family in which 10 of the 38 members who were exposed to general anesthetics developed fever and muscular rigidity. Approximately 200 cases of malignant hyperthermia with muscular rigidity have been reported, and the incidence is about one in 20,000 instances of general anesthesia (Kalow, 1972). The condition appears to be transmitted as an autosomal dominant, but great variability in the degree of muscular rigidity suggests genetic heterogeneity (Britt and Kalow, 1970; Kalow, 1971; Kalow, 1972). The body may become as stiff as a board, progressing without interruption into rigor mortis. The disorder may arise during anesthesia with halothane, succinylcholine, nitrous oxide, methoxy-

flurane, ether, cyclopropane, or combinations of these. Approximately two-thirds of the cases had ended fatally, usually with cardiac arrest. Consistently associated with malignant hyperthermia were tachycardia, tachypnea, hypoxia, respiratory and metabolic acidosis, hyperkalemia, and hypocalcemia (Britt and Kalow, 1970). It has now been discovered that intravenous procaine or procainamide can relieve the rigidity and fever in certain cases (Kalow, 1972). However, curare does not relieve the rigidity, nor will a limb under tourniquet become rigid, suggesting the existence of a peripheral rather than a central lesion in malignant hyperthermia. The underlying defect may involve the intracellular calcium stores in the skeletal muscles of affected subjects; these calcium stores may be abnormally inhibited by certain anesthetics in individuals subject to malignant hyperthermia (Kalow, 1972).

ATYPICAL LIVER ALCOHOL DEHYDROGENASE

A variant of the enzyme that metabolizes ethanol, alcohol dehydrogenase (ADH), has been described in man (von Wartburg and Schürch, 1968). Exceptionally active, the atypical enzyme is found in sufficiently high frequencies in Swiss and English populations to be designated a polymorphism. The variant occurred in 20% of 59 liver specimens from a Swiss population and in 4% of 50 livers from an English population.

A difference exists in the pH rate profiles so that the ratio of the activity at pH 10.8 to that at pH 8.8 is greater than 1 for the normal enzyme and less than 1 for the atypical enzyme. A chelator of zinc in the ADH molecule, *o*-phenanthroline, inhibits the normal more than the atypical ADH, whereas pyrazol inhibits the atypical more than the normal ADH. The normal ADH oxidizes the substrates *N*-butanol, benzyl alcohol, and cyclohexanol faster than the atypical ADH.

Three distinct peaks of both normal and atypical ADH occur on agar gel electrophoresis or ion exchange column chromatography (von Wartburg and Schürch, 1968; Blair and Vallee, 1966). With respect to the distribution of total ADH activity among the three bands, the ADH isozyme patterns of individual livers vary considerably; some livers contain only two bands. At pH 9.0 on agar gels the three normal and atypical ADH isozymes have approximately the same electrophoretic mobility (von Wartburg and Schürch, 1968).

What significant differences exist between the atypical and normal ADH with respect to the development of alcoholism remain to be established. These studies revealed a marked difference between the two enzymes in their rates of ethanol metabolism, but family studies should be performed to help elucidate the mode of inheritance of the trait.

Although exhibiting five- to sixfold more ADH activity than an equal amount of normal ADH in vitro, the atypical enzyme enhances alcohol metabolism by

only 40 to 50% in vivo, possibly because another factor such as reoxidation of coenzyme 1 becomes rate-limiting (von Wartburg and Schürch, 1968). The atypical ADH also reduces acetaldol (β-hydroxybutyraldehyde) five times faster than the normal ADH.

Attempts were made to correlate, after intravenous infusion of ethanol, rates of degradation of the drug with liver ADH typed from biopsies obtained at surgery (Edwards and Price Evans, 1967). Two of their twenty-three subjects had atypical ADH: in the male subject with atypical ADH, capacity to metabolize alcohol was no different from that in males with typical ADH, whereas in the female subject with atypical ADH, capacity to degrade ethanol was greater than in a small group of females who had typical ADH. The results of this study leave unresolved the question of whether individuals with atypical ADH possess increased capacity to degrade ethanol and, possibly, to resist alcoholic cirrhosis of the liver.

ETHANOL METABOLISM IN VARIOUS RACIAL GROUPS

Although it had been suspected for a long time that different racial groups varied considerably in their ability to metabolize ethanol and that individuals from Far Eastern countries possessed reduced ethanol-metabolizing capacity compared to subjects from Western countries, such impressions have not until now been satisfactorily documented. Furthermore, possible genetic differences might be obscured by elevated rates of ethanol metabolism as a result of chronic ethanol ingestion. This induction of ethanol metabolism by its chronic administration has been established by Lieber and De Carli (1968, 1970). A recent study has resolved many of the environmental factors influencing ethanol metabolism through a careful selection of subjects for the study which took into account differences in drinking habits (Fenna et al., 1971). Ethanol in a final concentration of 10% was infused intravenously in normal saline at 8:00 a.m. after an overnight fast. Infusions were stopped when a blood ethanol concentration of approximately 125 mg percent was attained. The study comprised 21 Eskimo, 26 Indian, and 17 white male volunteers. Some were patients in Canadian hospitals convalescing from fractures or acute infections. The rate of fall of blood ethanol among the whites (0.370 mg% per minute) was significantly greater than in either the Indians (0.259 mg% per minute) or Eskimos (0.264 mg% per minute). Furthermore, the rate of metabolism of ethanol was significantly greater in whites (0.1449 g/kg per hour) than in Indians (0.1013 g/kg per hour) or in Eskimos (0.1098 g/kg per hour). In each ethnic group a tendency to adaptation to ethanol was indicated, but only in Indians did this appear significant. However, neither previous intake of ethanol nor general diet appeared to be responsible for the faster rate of ethanol metabolism in whites, which was attributed to genetic factors (Fenna et al., 1971).

Additional evidence has recently been adduced to document racial differences in ethanol sensitivity. Wolff (1972) reported that Japanese, Taiwanese, and Koreans, after drinking amounts of ethanol that produced no detectable effect on Caucasians, exhibited marked facial flushing and mild to moderate symptoms of intoxication. Present since birth, these differences in ethanol responsiveness were attributed to variations in autonomic reactivity (Wolff, 1972).

CORRELATION OF CERTAIN GENETIC FACTORS WITH ADVERSE REACTIONS TO VARIOUS DRUGS

The approach of correlating certain genetic factors in individuals with their risk of contracting disease has resulted in the discovery of unusual ABO blood type distributions in patients with disorders such as gastric carcinoma, duodenal ulcer, rheumatic heart disease, pernicious anemia, and diabetes mellitus, among others (Vogen and Kruger, 1968). The reports from Jick and associates (Jick et al., 1969, and Lewis et al., 1971) have surveyed large populations of hospitalized patients (7000) in Boston to determine possible correlations between certain kinds of adverse reactions to drugs and various genetic factors for which 4500 of the patients have been typed. They discovered that in young women developing venous thromboembolism while on oral contraceptives there was a significant deficit of blood group O individuals relative to those possessing groups A and AB combined. Thus women of blood group O who take antifertility agents bear a significantly lower risk of developing a thromboembolism than do women of blood group A or AB. Possibly connected with this reduced liability to thromboembolism of individuals with blood group O are their reduced level of antihemophilic globulin in comparison to subjects with blood group A (Preston and Barr, 1964) and their increased risk of developing bleeding with peptic ulcers in comparison to ucler patients with A, B, or AB blood groups (Langman and Doll, 1965; Horwich et al., 1966). The Jick group (1972) reported a correlation between ABO blood groups and the development of arrhythmias after digoxin, with a decreased risk occurring in O group patients relative to non-O group patients.

The Boston group has also established a correlation between adverse reactions to prednisone and low serum albumin concentrations (Lewis et al., 1971). When albumin levels are low, the amount of prednisone that can be bound to this protein is correspondingly reduced and more of the drug is free and active so that the chances of side effects from the drug are increased.

Attempts at correlation of genetic factors to adverse drug reactions offer several possible applications. In addition to providing physicians with information on increased risk of drug toxicity in patients of certain genotypes, such correlations may furnish important clues to the pathogenesis of aberrant responses to drugs.

REDUCED DRUG-BINDING CAPACITY IN FETAL AND NEWBORN BLOOD

As an example of the need to resist the attribution to genetic factors of alterations in drug response produced by environmental changes, studies on the binding of sulfaphenazole to fetal, neonatal, and adult human plasma albumin are presented (Chignell et al., 1971). Fetal and neonatal albumin purified by electrophoretic separation and extensive dialysis exhibited reduced sulfaphenazole binding when compared to adult albumin prepared in a similar fashion. Although this different binding behavior might have suggested the existence of a structurally and hence genetically distinct fetal albumin, other experiments suggested that the binding properties resulted not from physiochemical differences in fetal and adult albumin but because in the fetus and for several months after birth albumin binds very avidly to certain compounds, probably either bilirubin, fatty acids, or both. After these compounds were removed by passing the albumin over charcoal, the binding properties of the albumin thus treated were identical to those of adult albumin (Chignell et al., 1971).

TOPICS IN THE THERAPY OF GENETIC DISEASE: NEW ASPECTS OF PHARMACOGENETICS

Differentiation of Two Genetically Specific Types of Depression by the Response to Antidepressant Drugs

Although sufficient data have not been gathered to establish this hypothesis, the interesting suggestion that depressive illness exists in at least two genetically different forms distinguishable by their response to imipramine and monoamine oxidase inhibitors is included here because it illustrates how drugs may aid in the diagnosis as well as the therapy of hereditary diseases. A well-accepted example is the administration of colchicine in cases of acute arthritis where gout is suspected; relief of the intense joint pain shortly after colchicine administration establishes a diagnosis of gout.

It has been suggested that the symptoms of depressive illness are produced by at least two genetically distinct entities and that "endogenous" depressions more frequently benefit from imipramine, whereas "reactive" depressions improve after monoamine oxidase inhibitors (Pare, 1970). Data supporting this hypothesis are disappointingly meager and are based on similarities in drug response between probands and relatives who also suffered from depression and who also received imipramine and monoamine oxidase inhibitors. The concordance in drug response among depressed relatives and depressed probands was reported to be statistically greater than expected by chance alone (Pare et al., 1962; Dally and Rhode, 1961). A more recent study in different patients tends to confirm these intial impressions (Pare and Mack, 1971).

Vitamin-Dependent Genetic Disease

A group of inborn errors of metabolism have recently been shown to respond not to physiologic replacement therapy but to pharmacologic doses of various vitamins (Rosenberg, 1970; Scriver, 1970; Mudd, 1971). Table 7 from Rosenberg (1970) shows nine of these disorders, their putative biochemical basis, their clinical manifestations, and the vitamin whose provision in very high dosage may prove therapeutic. Several mechanisms whereby the vitamin in pharmacologic doses could relieve the symptomatology of the disorder have been proposed. All of these mechanisms are based on the law of mass action according to which large concentrations of a vitamin overcome a genetically transmitted lesion in one of the steps involved in vitamin utilization by an organism. For example, if a defect existed in transporting the vitamin across a cell membrane to its site of action, this defective transport might exhibit improvement on furnishing very high, "saturating" concentrations of vitamin. If the lesion involved an enzyme responsible for converting a vitamin to its active form, as, for example, the metabolically inert vitamin B_6, pyridoxal or pyridoxine, is converted by a kinase in the presence of ATP to the biologically active coenzyme pyridoxal phosphate, then also a partial defect in the converting enzyme might be remedied by saturating the converting enzyme with very high concentrations of vitamin. If the disorder arose from alteration in the enzyme to which the vitamin in the form of a coenzyme is bound, then the avidity of binding might be increased by providing the enzyme with higher concentrations of coenzyme. There are evidently marked kinetic differences in behavior between lesions of type 2 ("leaky" mutants) and type 3 ("binding" mutants) (Mudd, 1971). Although in some of the disorders shown in Table 7 the precise mechanisms whereby pharmacologic doses of various vitamins are clinically effective remain to be established, some progress has been made. For example, in vitamin B_{12}-responsive methylmalonic aciduria, the primary defect has been determined; it is a partial defect in the biosynthesis of 5′-deoxyadenosylcobalamin, one of the three naturally occurring forms of cobalamin in mammalian tissue (Mahoney and Rosenberg, 1970). In three of the B_6-responsive genetic diseases shown in Table 7 in which the enzyme deficiency is known (cystathioninuria, xanthurenic aciduria, and homocystinuria), the affected enzyme normally is activated by pyridoxal phosphate. Certain individuals with B_6-responsive cystathioninuria or xanthurenic aciduria possess a mutant apoenzyme that binds pyridoxal phosphate abnormally; this lesion in the binding of apoenzyme and coenzyme can be substantially overcome by providing large concentrations of coenzyme. In homocystinuria, on the other hand, therapeutic improvement from vitamin B_6 administration appears not to result from restoration of the activity of the defective enzyme, cystathionine synthase, to near-normal levels since the low activity of this enzyme isolated from B_6-responsive homocystinuric patients is not altered in vitro by addition of large amounts of vitamin B_6 (Mudd, 1971). In many inborn errors of metab-

TABLE 7.–*Vitamin-Dependent Inborn Errors of Metabolism**

Disorder	Clinical manifestations	Vitamin required	Vitamin dose		Biochemical basis
			Normal requirement	Patient requirement	
Thiamine-responsive megaloblastic anemia	Magaloblastic anemia	Thiamine (B_1)	1 mg/day	20 mg/day	Unknown
Hartnup's disease	Intermittent cerebellar ataxia; mental retardation	Nicotinamide	5-10 mg/day	40-200 mg/day	Defective intestinal absorption of tryptophan
B_6-dependent infantile convulsions	Clonic and tonic seizures	Pyridoxine (B_6)	1-2 mg/day	10-25 mg/day	Defective glutamic acid decarboxylase (?)
B_6-responsive anemia	Microcytic, hypochromic anemia	Pyridoxine (B_6)	1-2 mg/day	>10 mg/day	Defective δ-amino levulinic acid synthetase (?)
Cystathioninuria	Probably none	Pyridoxine (B_6)	1-2 mg/day	200-400 mg/day	Defective cystathionase
Xanthurenic aciduria	Mental retardation (?)	Pyridoxine (B_6)	1-2 mg/day	5-10 mg/day	Defective kynureninase
Homocystinuria	Ectopia lentis; arterial and venous thromboses; mental retardation	Pyridoxine (B_6)	1-2 mg/day	25-500 mg/day	Defective cystathionine synthetase
Methylmalonic aciduria	Infantile ketoacidosis; developmental retardation	Cobalamin (B_{12})	1 μg/day	200-1000 μg/day	Defective biosynthesis of B_{12} coenzyme
Familial hypophosphatemic rickets	Rickets; short stature	Calciferol (D)	400 units/day	50,000-200,000 units/day	Unknown

* From Rosenberg (1970).

olism it is important to recognize when attempting to devise new therapeutic maneuvers that only a very slight increase in the activity of a defective enzyme can produce substantial clinical improvement. Since an enzyme functions as a catalyst, only a small change in its catalytic efficiency can produce a large alteration either in elevating the concentration of a necessary product of the reaction or in reducing the amount of a toxic metabolite.

Heterogeneity of Hyperlipidemia: Relationship of Therapy to Genetic Classification

Multiple genetic and environmental factors can produce hyperlipidemia. Therefore, hyperlipidemia is not a specific disease entity but a symptom of a heterogeneous group of disorders differing in etiology, clinical manifestations, prognosis, and response to therapy (Levy and Langer, 1971; Levy and Fredrickson, 1970; Levy and Fredrickson, 1968). The requirement for different therapies in hyperlipidemias that have different genetic bases illustrates the important pharmacogenetic principle that treatment of a biochemical abnormality may change significantly depending on the genetic etiology of the defect so that the extent and nature of the genetic heterogeneity of a biochemical aberration may have profound therapeutic implications.

Levy and Fredrickson (1968, 1970) divide plasma lipid abnormalities into five different types according to the mobility of the beta and pre-beta lipoproteins on paper electrophoresis. Each of these types can be produced by several different pathogenetic mechanisms and hence can be exhibited in various disorders, some acquired, others hereditary in nature. For example, type II may arise from increased synthesis of beta lipoproteins, as in porphyria, or decreased catabolism, as in myxedema (Levy and Langer, 1971). The five types define the location of the problem in the complex picture of lipid transport. For example, patients with type I have a defect in handling dietary fat, whereas in type II the problem involves the metabolism of beta lipoproteins. In type III aberrant beta-lipoprotein bands appear. In type IV endogenous glycerides are abnormally handled, and in type V both exogenous and endogenous triglycerides are assimilated with difficulty (Levy and Langer, 1971; Levy and Fredrickson, 1968).

When acquired disorders are eliminated as causes of the five types of abnormal lipoprotein patterns, then the following modes of transmission, clinical characteristics, and therapies of primary hyperlipoproteinemia have been suggested (Levy and Langer, 1971; Levy and Fredrickson, 1970):

Type I, caused by defective removal of dietary fat in chylomicrons from the blood, is transmitted as a rare autosomal recessive and possibly involves deficiency of normal lipoprotein lipase activity. The patients present clinically, generally in early childhood, with lipemia retinalis, eruptive xanthomas, hepatosplenomegaly, abdominal pain, and pancreatitis. At present no drugs appear effective, and diet consists in restriction of fat to approximately 25 g per day supplemented with medium-chain-length triglycerides.

Type II, the most common form of hyperlipoproteinemia, is associated with decreased catabolism of beta lipoproteins and is transmitted in certain families as an autosomal dominant. Expression of the disorder is variable and generally much less severe when only a single abnormal gene is present. Affected individuals, usually detected before the age of 20, exhibit tendon and tuberous xanthomas, corneal arcus, xanthelasmas, and often fatal atheromatosis. Therapy with cholestyramine, 16 to 32 g per day, is effective, but D-thyroxine and nicotinic acid are also useful. The diet should be low in cholesterol (less than 300 mg per day) and saturated fat, but should be supplemented by an increased intake of polyunsaturated fats.

Type III is similar in some respects to type II, but the hypercholesterolemia is accompanied by hyperglyceridemia of an endogenous or carbohydrate-induced nature. In some cases, it appears to be transmitted as an autosomal recessive. As in type II, the defective gene product is unknown. Type III, generally manifested in adults, is characterized by abnormal glucose tolerance, tuberous and planar xanthomas, and early ischemic heart disease. In conjunction with dietary restrictions, clofibrate, 2 g per day, is uniformly effective. D-Thyroxine and nicotinic acid are also effective. Reduction to normal body weight and a low-cholesterol (less than 300 mg per day) balanced diet (40% of calories as fat and 40% as carbohydrate) are recommended.

Type IV exhibits increased prebeta lipoproteins and consists of an endogenous, carbohydrate-induced hyperlipemia. The beta lipoproteins are not elevated and plasma cholesterol is usually normal. Transmission has been shown in certain families to be autosomal dominant. The specific biochemical lesion is unknown, but may involve excessive endogenous glyceride synthesis or deficient glyceride clearance. Affected individuals, generally adults, may exhibit accelerated coronary artery disease, abnormal glucose tolerance, and hyperuricemia. Therapeutically clofibrate may be useful, but in some cases may be less effective than in type III. Nicotinic acid, 3 to 6 g per day, is recommended. In addition to a return to normal body weight, an increased intake of polyunsaturated fats and restriction of carbohydrates and alcohol are suggested.

Type V is a complex, familial disorder involving severe hyperlipemia of both endogenous and exogenous origin. The mode of inheritance and the primary biochemical defect remain to be established. There are excesses of chylomicrons and prebeta lipoproteins. The disease, seen in early adulthood, is characterized by lipemia retinalis, eruptive xanthomas, hepatosplenomegaly, abdominal pain, hyperglycemia, and hyperuricemia. Therapy consists of nicotinic acid (3 to 6 g per day). As in type IV, clofibrate appears to be less efficacious than in type III. Management includes weight reduction and a diet increased in protein, but low in fat and carbohydrate.

Drugs used to treat hyperlipidemia apparently act on the rates of synthesis and catabolism of lipoproteins. For example, cholestyramine increases the

catabolic rate of lipoproteins, whereas nicotinic acid decreases their rate of synthesis (Levy and Langer, 1971). This mechanism of action is logical since by definition cholesterol, phospholipid, and triglycerides are insoluble in aqueous solutions and cannot circulate freely in the blood. These fats depend on attachment to proteins for their solubility in blood; therefore, a rational means of reducing concentrations of fat in cells is to reduce the concentrations of proteins on which fats depend for their transport to tissues.

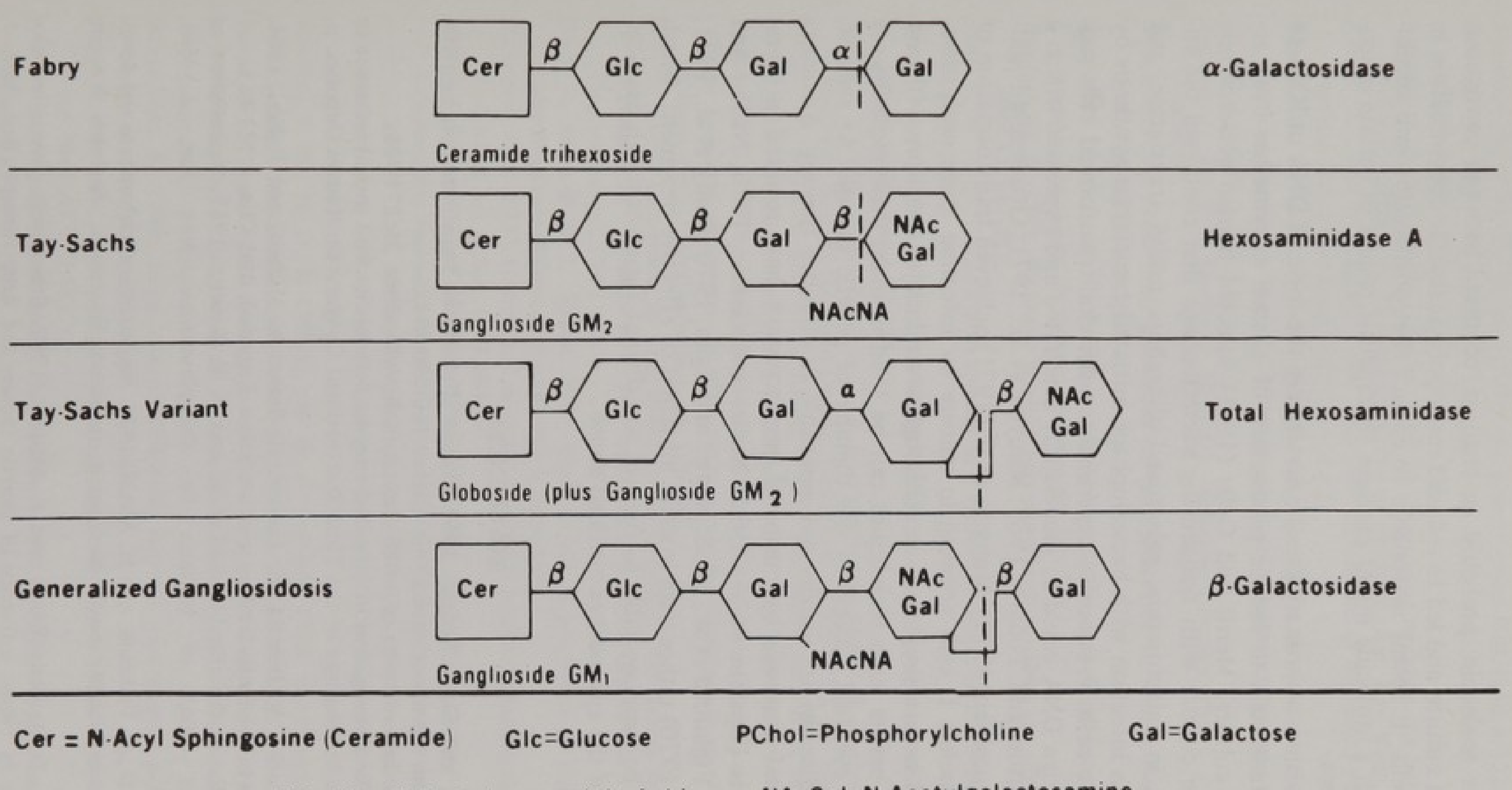
Genetic studies should be performed on many additional families with hyperlipidemia to establish the mode of inheritance of the more frequently encountered varieties of this abnormality. A principal of human genetics states that it is particularly difficult to define the mode of inheritance of disorders found in high frequency.

Therapy of Inborn Errors of Metabolism

The lipid-storage diseases listed in Table 8 (from Brady, 1971) are typical in their illustration of the several successive stages that frequently occur in the accumulation of knowledge concering inborn errors of metabolism. After the clinical description of the syndrome, chemical identification of the compounds stored in abnormal quantities in various tissues is generally achieved. Elucidation of the nature of the metabolic defect is a frequent next step. Diagnostic techniques to define affected individuals and separate them from carriers are usually developed. Finally, attempts at therapy are undertaken. Although thus far these have been disappointingly unsuccessful in most inborn errors of metabolism, several recent advances give promise of eventual therapeutic success in some genetic disorders. Brady (1971) believes that once the requisite enzymes are prepared in sufficient purity, attempts at enzyme replacement as therapy for lipid-storage diseases could take the form of parenteral administration of purified enzyme from human or animal sources or of native or polymer-stabilized enzyme encapsulated in biodegradable microspherules. Alternatively the need for enzyme purification would be obviated if organ transplantation could supply normal enzyme to deficient subjects. It seems unlikely that exogenously administered enzymes could reach sufficient concentrations to be effective within the cells where they are required, and treatment of metachromatic leukodystrophy with arylsulfatase A has been unsuccessful (Greene et al., 1969). Another approach would involve passage of a patient's plasma over columns or sheets of stably bound enzyme to reduce the level of circulating sphingolipid, thereby permitting removal of intracellular accumulations (Brady, 1971). A potentially therapeutic approach is suggested by the observation that in certain mucopolysaccharidoses growth of cells from two genetically distinct forms of the disease in the same environment results in a correction of each enzymatic defect (Fratantoni et al., 1969; Danes and Bearn, 1970; Neufeld and Cantz, 1971).

TABLE 8.–*Constellation of Metabolic Diseases Characterized by Inability to Degrade Sphingolipids**

Disease	Major sphingolipid accumulated	Enzyme defect
Gaucher	Cer –β– Glc Ceramide glucoside (glucocerebroside)	β-Glucosidase
Niemann-Pick	Cer – PChol Sphingomyelin	Sphingomyelinase
Krabbe	Cer –β– Gal Ceramide galactoside (galactocerebroside)	β- Galactosidase
Metachromatic Leukodystophy	Cer –β– Gal – OSO_3^- Ceramide galactose-3-sulfate (sulfatide)	Sulfatidase
Ceramide Lactoside Lipidosis	Cer –β– Glc –β– Gal Ceramide lactoside	β-Galactosidase



*From Brady (1971).

Passage of some molecule, probably an enzume, occurred between contiguous cells growing in culture and led to reduced mucopolysaccharide accumulation in the defective cells. It seems reasonable to expect that purification and administration of such a molecule might produce therapeutic results in certain mucopolysaccharidoses.

Finally, provision of primary genetic material in the form of DNA or RNA to a patient's mutant cells to induce production of normal enzyme has been accomplished in culture. Merril and Geier (1971) infected human galactosemic cells growing in culture with transducing bacteriophage that contained the enzyme deficient in galactosemia, galactose-1-phosphate uridyl transferase, and showed that this infection with bacteriophage initiated transferase synthesis by the galactosemic cells. Previously, experiments with viral-transformed cells suggested that foreign DNA could be successfully integrated and expressed within a cell line (Aaronson and Todaro, 1969; Munyon et al., 1971; Ottolenghi-Nightingale, 1969; Aposhian, 1972). Munyon et al. (1971) achieved transformation of thymidine kinaseless L cells growing in culture by infecting them with ultraviolet-irradiated herpes simplex virus. Although several major questions will have to be resolved before such techniques can be tried in man, these experiments offer hope for eventual success in the therapy of genetic disease. At present, severe genetic diseases are usually not amenable to effective therapy. Advances in their antenatal diagnosis by means of amniocentesis have resulted in therapeutic abortions (Epstein et al., 1971; Nadler and Gerbie, 1970; Nadler and Messina, 1969; Fujimoto et al., 1968; Nadler and Egan, 1970; Brady et al., 1971; Schneck et al., 1970) rather than in early initiation of effective treatment, which hopefully may become available in the not too distant future through development of some of the approaches outlined above.

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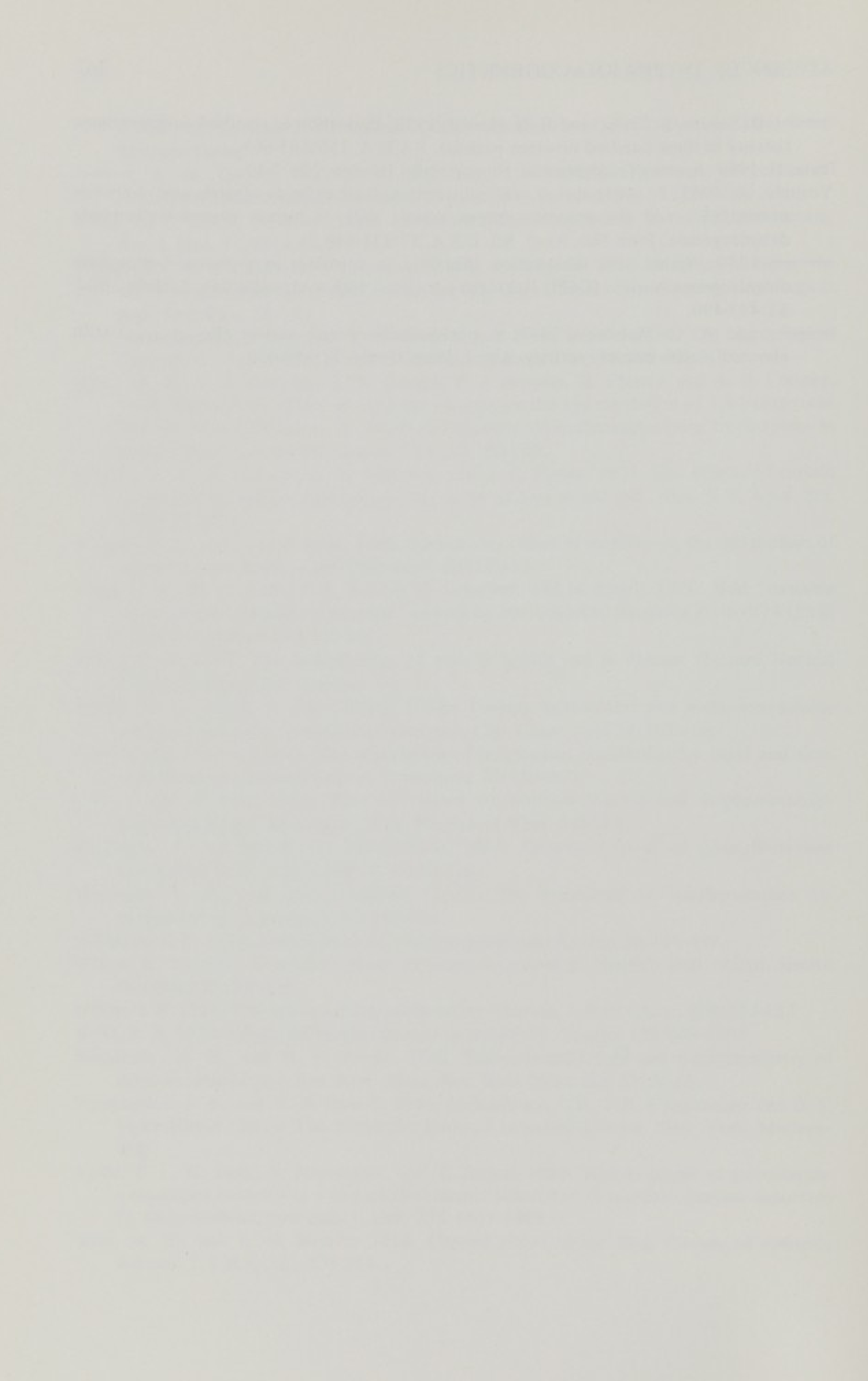

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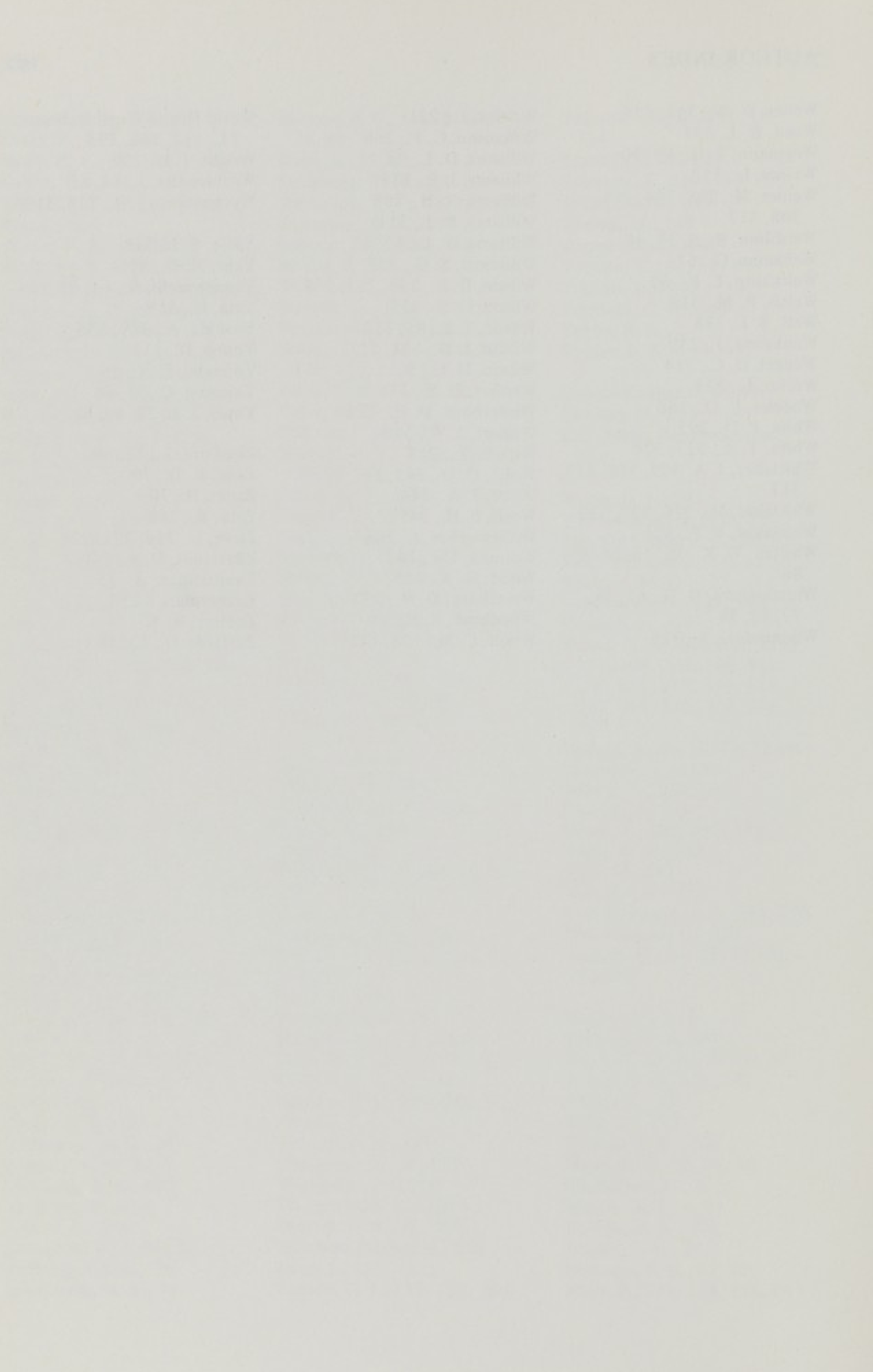

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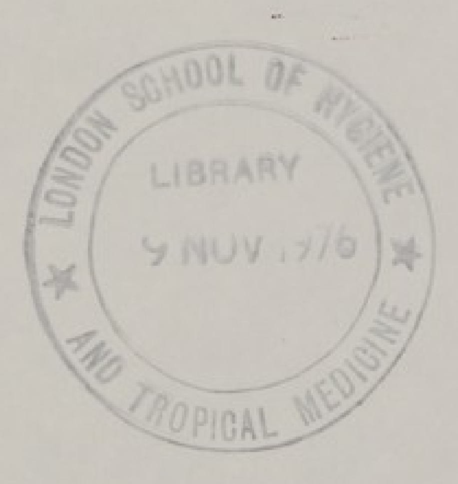
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