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Progress in

MEDICAL GENETICS

VOLUME X

Edited by

ARTHUR G. STEINBERG

ALEXANDER G. BEARN

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Francis Hobart Herrick Professor of Biology, Department of Biology, and Professor of Human Genetics, Department of Reproductive Biology, Case Western Reserve University, Cleveland, Ohio

and

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Contents

	Foreword	vii
1.	The Mutation Rate in Man *John Edwards*	1
2.	Biochemical Polymorphisms in Animals as Models For Man *Michael J. Siciliano, David A. Wright, and Charles R. Shaw*	17
3.	Inborn Errors of the Thyroid *John B. Stanbury*	55
4.	The Biochemical Basis for Mucopolysaccharidoses and Mucolipidoses *Elizabeth F. Neufeld*	81
5.	Approaches to the Control and Prevention of Tay-Sachs Disease *Michael M. Kaback, Robert S. Zeiger, Linda W. Reynolds, and Marguerite Sonneborn*	103
6.	The XYY Chromosome Male—or Syndrome *Digamber S. Borgaonkar and Saleem A. Shah*	135
7.	Ethical Issues in Genetics *Robert M. Veatch*	223
	Author Index	265
	Subject Index	271

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	Foreword	vii
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Foreword

During the past year, the surge of activity in the field of human genetics has continued unabated. The selection of topics discussed in the present volume of *Progress in Medical Genetics* represents but a small sample of this expanding area of biomedical science.

Although mutation rate in man was first discussed more than 30 years ago, John Edwards brings fresh insight and a new approach to this important topic (Chapter 1). His article is particularly timely because the evident threat of chemical mutagenesis assumes increasing importance in our highly technological society. Biochemical polymorphism is a widespread phenomenon in man and other species. Michael J. Siciliano, David A. Wright and Charles R. Shaw address themselves in a most interesting fashion to the occurrence of polymorphism in different species with particular reference to the applicability of animal models in the study of human polymorphism (Chapter 2). Inborn errors of the thyroid have become increasingly well recognized as the enzymatic reactions governing the translation of iodine into thyroid hormones are better understood. These newer studies are illuminatingly discussed by John B. Stanbury whose extensive experience with inherited abnormalities of thyroid metabolism is summarized in Chapter 3.

During the past several years the imaginative research of Elizabeth F. Neufeld has brought clarity and understanding to the several inherited mucopolysaccharidoses. These diseases have been disentangled and placed on a molecular basis largely as a result of her painstaking work (Chapter 4). No longer is there any doubt that screening for inborn errors of metabolism is becoming increasingly important. The pioneering studies of

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The observation that XYY chromosome constitution can occur in man has raised a number of fascinating biological, legal, and social questions. The present state of our knowledge of this chromosome abnormality is informatively discussed by Digamber S. Borgaonkar and Saleem A. Shah in Chapter 6. Ethical issues in Genetics have been a source of lively debate in recent years as amniocentesis and selective abortion have become increasingly available. Robert M. Veatch addresses himself to many of the current ethical questions in human genetics in the concluding chapter of this volume (Chapter 7).

The Editors wish to express their deep gratitude to the authors of this volume of Progress in Medical Genetics. Their perceptive and scholarly articles will do much to acquaint our readers with the broad significance of the science of genetics in health and disease.

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1

The Mutation Rate in Man

J. H. Edwards

Infant Development Unit Birmingham Maternity Hospital
Birmingham 15, England

INTRODUCTION 2
THE NATURE OF THE GENETIC APPARATUS 2
CLASSICAL METHODS 5
OTHER METHODS 7
Direct Studies on Phenotypes 7
Direct Studies of Markers 8
Theoretical Arguments 10
Estimates from Other Species 11
Special Methods 11
CONCLUSIONS 12
REFERENCES 15

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INTRODUCTION

By mutation we mean *change*, and by mutation rate we mean the *rate of change*. But what changes? This is the fundamental problem which, for historical and technical reasons, remains unanswered, in the sense that in man, as in other mammals, we are almost devoid of information on the mutation rate of defined units. From substantial and consistent studies investigators have estimated the rate at which recurrent novelties arise *de novo*, and some of these novelties belong to consistent diagnostic categories, such as hemophilia, Marfan's syndrome, or achondroplasia, in which the condition can occur spontaneously, and having once occurred, it can be passed on in a consistent way. This development of novelties has long been known, and systematic estimates of such effects have been made in many species. The purpose of this chapter is to present the problem in a deductive context, based on what may be regarded as the orthodox view of genetic determinants, and to consider what is known, and what can be known, of the mutation rate in our own species, restricting the term to conditions in which the nature of the mutating unit has been defined.

No attempt will be made to duplicate the extensive reviews appearing as chapters in Hollaender's "Chemical Mutagens" (1971) or in Vogel and Röhrborn's "Chemical Mutagenesis in Mammals and Man" (1970).

THE NATURE OF THE GENETIC APPARATUS

In mammals there is a hierarchy of genetic elements, each of which is capable of undergoing spontaneous change at the time of its reproduction (Fig. 1). At the coarsest level we may consider the whole cell, which frequently divides improperly to produce a tetraploid nucleus, a condition normal in some tissues, inconsistent and harmless in most. This is a somatic event and will not be considered further.

The largest true hereditary unit, the haploid set of chromosomes, is liable to erratic replication or partition, and in man triploidy is the commonest known cause of death between conception and puberty, with an incidence of about 1 percent. Almost all mutants perish as early abortuses (Carr, 1965; Edwards et al., 1967).

The next largest unit of heredity, the single chromosome, is also liable to misplacement at division, and almost 1 percent of conceptions suffer, usually fatally, from these mutations. Some surviving forms, such as trisomy 21 in man (mongolism or Down's syndrome) and X0 monosomy in mice, are true genetic mutants in the sense of being transmissible. Trisomy 21 is a dominant mutation, in the classical sense, and its rate is capable of accurate estimation averaging rather more than half its incidence, or almost 10^{-3} with a range,

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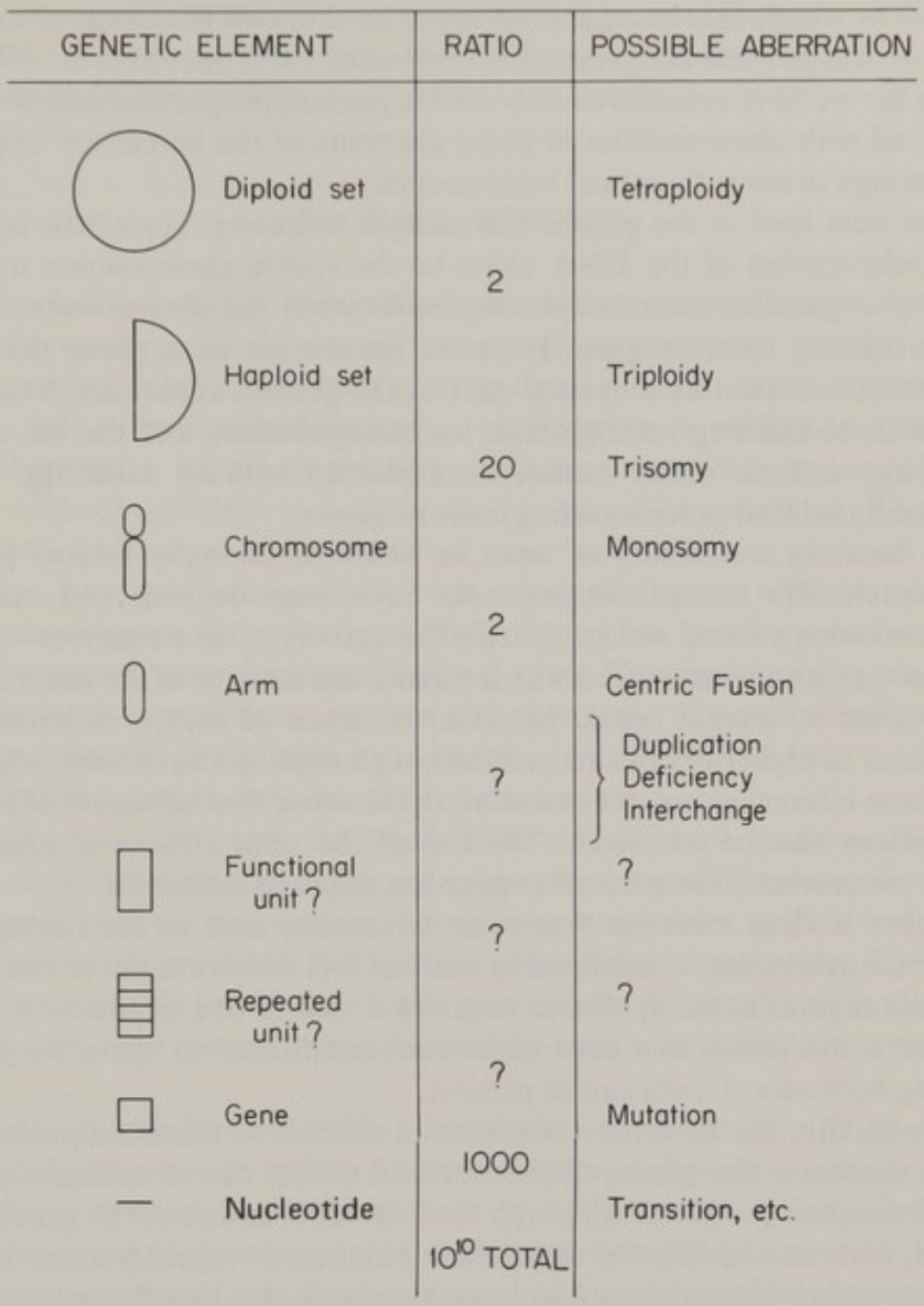
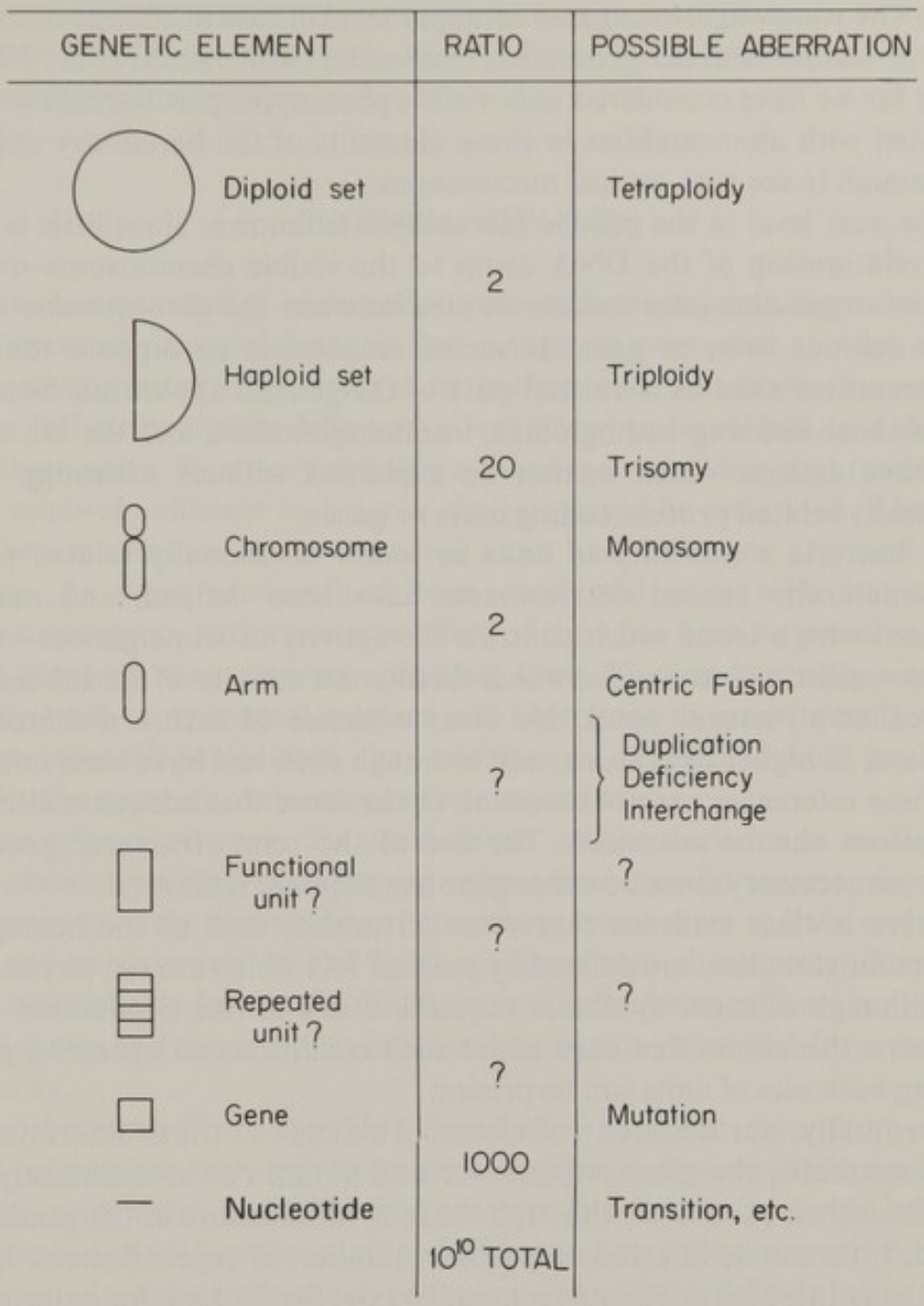


Fig. 1. Hierarchy of genetic elements.

depending primarily on maternal age, of 10^{-4} to over 10^{-2}. As with most mutations, there is reason to suspect an increase in mutation rate from exposure to X-rays (Uchida et al., 1968).

Parts of chromosomes, the chromosome arms, or parts of these, are liable to rearrangement, balanced forms being harmless. Since segregation is usually such as to favor the balanced forms, estimates of rate are difficult. Various forms have been described with population incidences of up to 10^{-3} and the incidence of unbalanced forms is certainly less than 10^{-4}. In most cases with balanced translocations, the translocation is also present in a



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parent. The rough figures suggest an upper level of rate of change of the order of 10^{-4} or less per unit per generation (Edwards, 1970; Jacobs et al., 1972).

So far we have considered only visible phenotypic peculiarities which are associated with abnormalities in those elements of the hereditary apparatus large enough to see with optical microscopes.

The next level in the genetic hierarchy is unknown, since little is known of the relationship of the DNA chain to the visible chromosome or of the nature of organelles intermediate in size between the chromosome and the protein-defining unit, or gene. It seems reasonable to suppose that some such structures exist as a normal part of the genetic apparatus. Some loci, such as those defining hemoglobins, immunoglobulins, and the HL-A antigens, have actions which cannot be explained without assuming several structurally related protein-coding units or genes.

In bacteria a minority of units in which functionally related proteins have structurally related determinants have been defined, and many are associated with a locus which controls the activity of its neighbors—the site of the so-called operator gene which directs the activity of its subordinates, the so-called structural genes. No clear evidence of such a dichotomy has been found in higher organisms, and although such loci have been inferred in man, these inferences are not essential, in the sense that adequate alternative explanations can be advanced. The use of the term *structural gene* is, at present, unnecessary since no other gene has required definition.

There is clear evidence that some tetramers, such as the hemoglobins and immunoglobulins, are defined by pairs of loci which are not physically related although efficient synthesis requires a one-to-one production rate of monomers; this shows that even under such conditions no operative gene integrating both sets of units can be present.

Eventually, our heirarchy of elements extends to those determinants of protein synthesis, the genes, whose size and nature can so accurately be inferred from their products. Although the individual nature of the genes can be inferred, little can be inferred about their number, or repetitiveness. In those few proteins in which adequate techniques exist for looking for heterogeneity at the amino acid level, it has been found; this suggests that it may be a common, or even a general, property (Harris, 1969, 1971, 1973).

If we accept the simple model of the gene as a series of $a + 3b$ units, or nucleotides, coding for b amino acids (where a is the number of nucleotides needed for other purposes), then we can make some rough estimates. The DNA in man is sufficient for about 3×10^9 units per haploid set. An average protein with 2 to 4×10^2 amino acids requires about 10^3 nucleotides, leaving room for about 3×10^6 genes. Ribosome production, chromosome structure, spacing, duplication, and other unknown features of chromosomal organization may reduce the number of distinct units to 10^6 or even less. The estimates of the numbers of repeating sequences from annealing and allied studies involve very indirect inferences but are consistent with as many as 10^6 units.

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If we accept a genetic hierarchy with these general features, we may ask how could mutations be evident, and how could inferences about mutation rate be made by observation.

CLASSICAL METHODS

The classical studies of mutation rate were made when the concept of a genetic hierarchy was unclear, so that a vaguely connected nuclear mass of discrete units limited in numbers to mere thousands was implicitly assumed, and any self-perpetuating defect was considered to be related to a defect in one of these units. By simply defining a balance sheet of loss and renewal, the rate of renewal sufficient to balance the loss could be estimated. These estimates became known as the *mutation rates* and have now achieved a wide currency. As most studies had been conducted at a high standard by geneticists of great distinction, the effect of time has been to preserve, rather than to question, their relevance. Indeed, some 20 years later they were used to justify both experimental warfare and objections to experimental warfare and now, over 30 years later, they continue to be used to both justify and denigrate the economic advantage of nuclear power.

This is very strange, for when these studies were made nothing was known about what was mutating, nor about how it could mutate. A priori, it might seem unlikely that studies of structures which were so little understood would not need review in the light of subsequent discoveries. The major studies were of two forms: the direct, in which the balance sheet is closed, as in dominants, and the indirect, in which the rate is estimated from the effect on viability.

Gunther and Penrose (1935) found that the birth rate, from normal parents, of persons with epiloia (tuberous sclerosis; Bourneville's syndrome), a condition in which peculiar structures arise in the brain, lungs, and skin, often associated with severe mental deficiency with epilepsy, was 1.6×10^{-5}. Numerous surveys of the more common and most severe dominants have given similar values. This is not altogether surprising, for the method depends on both commonness and severity, and only those conditions so common and severe that they are both defined by textbooks and available for study can be used.

A dozen or so conditions fall into this category. Several hundred rarer dominants necessarily have lower mutation rates, and taken together, all these dominants give an incidence of a severe dominant per generation of 10^{-3} to 10^{-4}. Clinical experience would suggest the former frequency is far too high since the frequency at birth can hardly exceed 10^{-3}. An ingenious approach to estimating the bias involved by such studies (Cavalli-Sforza and Bodmer, 1971) suggests an error of approximately tenfold.

In X-linked disorders the commoner severe conditions appear to be

mutating at around 10^{-5} per individual per generation. Duchenne's muscular dystrophy shows a mutation rate approaching 10^{-4} in some series. Cumulatively, these disorders lead to 10^{-3} to 10^{-4} per individual per generation, probably nearer 10^{-4}.

Attempts have been made to extend the indirect method to recessive conditions. This is difficult, as these are largely racially restricted, the effect of isolation and race formation being to concentrate certain forms: this is because when the group had only n ancestors, the gene frequency must have been $0/2n$, $1/2n$, $2/2n$, and so forth, and if it was not zero, it must have been large if n was small. The effect is such that although the total incidence of recessive lethals will be conserved, the variation in their incidences will be increased.

Mutation rate can have little part in maintaining the present levels, which reflect ancestral frequencies and are largely fortuitous. It is likely that most cases of the many recessive disorders in man are descendants of a single person, and this will be clear as soon as the offending proteins have had their amino-acid sequences laid bare. No precise evidence or information on mutation rates is available from studies of recessive disease in man so that our total evidence from studies of disease is limited to severe dominant and X-linked disorders, but the relevance of these rates to mutations at the nucleotide level is obscure.

In the case of the so-called dominants, there is no doubt that they are very specific clinically: although diagnostic problems arise, 70 percent of patients with achondroplasia, Marfan's syndrome, and epiloia are clearly homogeneous, and only mildly influenced by the varying genetic backgrounds imposed by sex and race. If the problems are caused by the positive activities of some abnormal protein, then it seems this would have to be a very specific peculiarity. An average protein has around 10^3 determining nucleotides, each of which can change in three ways, though some changes may be synonymous on translation. We can reasonably suggest that not more than 1 in a 1,000 changes would have a result of such specificity. In this case the mutation rate per nucleotide would be around 10^{-8}. As there are 3×10^9 nucleotides per gamete (based on chemical estimates of Boivin et al., 1948), we would expect 60 mutations per generation if such mutation rates were widespread; this is impossible, for there is no mechanism for clearing mutants at such a rate.

We must suppose that either these loci are peculiar, or "hot," in which case we cannot extrapolate, or that they are not loci in the specific protein-determining sense, in which case again we cannot extrapolate. What is mutating is not clear, although it seems possible that the increased definition now possible with specific chromosome straining might reveal some peculiarity, as in the so-called Bar locus in drosophila.

The mutation rate in X-linkage is similarly incapable of reflecting a typical nucleotide change. Here the matter is less obscure, for the X chro-

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Mutation rate can have little part in maintaining the present levels, which reflect ancestral frequencies and are largely fortuitous. It is likely that most cases of the many recessive disorders in man are descendants of a single person, and this will be clear as soon as the offending proteins have had their amino-acid sequences laid bare. No precise evidence or information on mutation rates is available from studies of recessive disease in man so that our total evidence from studies of disease is limited to severe dominant and X-linked disorders, but the relevance of these rates to mutations at the nucleotide level is obscure.

In the case of the so-called dominants, there is no doubt that they are very specific clinically: although diagnostic problems arise, 70 percent of patients with achondroplasia, Marfan's syndrome, and epiloia are clearly homogeneous, and only mildly influenced by the varying genetic backgrounds imposed by sex and race. If the problems are caused by the positive activities of some abnormal protein, then it seems this would have to be a very specific peculiarity. An average protein has around 10^3 determining nucleotides, each of which can change in three ways, though some changes may be synonymous on translation. We can reasonably suggest that not more than 1 in a 1,000 changes would have a result of such specificity. In this case the mutation rate per nucleotide would be around 10^{-8}. As there are 3×10^9 nucleotides per gamete (based on chemical estimates of Boivin et al., 1948), we would expect 60 mutations per generation if such mutation rates were widespread; this is impossible, for there is no mechanism for clearing mutants at such a rate.

We must suppose that either these loci are peculiar, or "hot," in which case we cannot extrapolate, or that they are not loci in the specific protein-determining sense, in which case again we cannot extrapolate. What is mutating is not clear, although it seems possible that the increased definition now possible with specific chromosome straining might reveal some peculiarity, as in the so-called Bar locus in drosophila.

The mutation rate in X-linkage is similarly incapable of reflecting a typical nucleotide change. Here the matter is less obscure, for the X chro-

mosome is known to be particularly prone to nondisjunction and isochromosome formation; other structural changes, leading to small deficiencies, may reasonably be suspected. The matter should be clear when the clotting factors necessary to prevent the hemophilias (hemophilia and Christmas disease) have been isolated and sequenced in both health and disease.

The position, then, is that we have mutation rates relating to phenotypes defined by unknown units, and we have well-defined units whose mutation rates are unknown. We know virtually nothing of the human mutation rate beyond the constraints imposed by certain theoretical arguments. (For a summary see Kimura, 1970; Kimura and Ohta, 1971.) Even these methods involve uncertainties, in the sense that vindication by computer simulation has so far been restricted to small populations whose number is of the same order as the reciprocal of the postulated mutation rates.

OTHER METHODS

Direct Studies on Phenotypes

The number of recessive lethals in a population may, in principle, be estimated from the regression of incidence on inbreeding (Morton et al., 1956). The theoretical basis implicitly assumes that the inbred and outbred subsets of the population studied are freely interbreeding units. There is no theoretical expectation of any regression of incidence on inbreeding within closed systems; this is obvious since the stability conditions of recessives demand that the gain by mutation should equal the loss through homozygosity.

Various attempts have been made to estimate the total number of recessive alleles per gamete, and, notwithstanding the wide scatter of estimates (for reviews see Fraser, 1962; Cavalli-Sforza and Bodmer, 1971), it is usually asserted that most gametes convey one, or even several, recessive lethals.

A more direct approach is the enumeration of disorders with due reference to estimates of the proportion of genic causes in the deaf (Fraser, 1965), the blind (Fraser and Friedmann, 1967), and the defective (Penrose, 1938). The data are limited, but estimates so derived can hardly be much rougher than those from load arguments, as based on Muller's concept (Muller, 1956) of a "load" imposed on the viability of populations by the occurrence of mutations. Tables 1 and 2 are based on an experience of working with genetic and congenital disorders in a central area of England which has unusually good records, especially in its main city (Leck et al., 1968). No attempt has been made to include recent immigrant populations. There is considerable Irish admixture, possibly about 10 percent, and substantial contributions from Wales and Scotland. The Jewish contribution is small.

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The major unknown is the extent to which clinical disorders which resist

resolution are determined at different loci; here again estimates, although often quoted with precision, are very rough. It is convenient to introduce the term *allelic equivalents*, by which is meant the number of lethal alleles, at distinct diallelic loci with equal allelic proportions (or gene frequencies), which would show the same sum of both mutant alleles and mutant recessive phenotypes. That is, if the underlying allelic proportions are

$$p_1, p_2, p_3, \ldots,$$

and so forth, so that the expected proportions of recessive phenotypes are

$$p_1^2, p_2^2, p_3^2,$$

then the allelic equivalent is a number, n, such that

$$\Sigma \left[\frac{(\Sigma p_i)}{n} \right]^2 = \Sigma p_i^2$$

and, when all allelic proportions are equal, is equal to the number of loci.

On the rather personal rough estimates in Table 1 it seems that the number of lethals per gamete may well have an expectation of considerably less than unity, but almost certainly more than a quarter.

Note that, in childhood, recessive disorders are far more evident than dominant disorders. This must be accepted, notwithstanding numerous statements to the contrary. Some very much higher estimates of genic disorder are in circulation: these frequently include congenital disorders for reasons which are unclear.

Direct Studies of Markers

If a marker is defined by 10^3 nucleotides and, of the 3×10^3 different nucleotides possible by single-step mutants, 10^3 can be detected, we could search a series of families for new proteins. This procedure has the attraction that automated methods will allow numerous proteins to be analyzed, and that liquid-nitrogen storage of red cells, serum, and even living lymphocytes can allow further scrutiny as new techniques are discovered.

Even here there are difficulties, except in closed communities within which mating is restricted, in which there is a complete census and in which almost everyone is sampled. This is difficult to do unless blood is routinely acquired at birth and at death, the only times at which it can be acquired without consent, hazard, or discomfort. (Venipuncture in babies is not devoid of risk.) Blood is easily and harmlessly acquired from the placental end of the cord at birth.

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Table 1. Hereditary Disorders in Man: Genic

Disorder	NA*	Number per million	Recurrent per million	Heterozygotes per 1000
Autosomal recessives				
Fibrocystic disease	1	500	100	50
Adrenogenital syndrome	2	100	20	20
Deafness, all forms, severe	16	500	100	150
Blindness, all forms, severe	16	200	40	100
Mental defect, severe ex. A.A.†	16	800	160	50
Albinism	2	100	20	20
Phenylketonuria	1	100	20	20
Other harmful and treatable A.A.	4	50	10	15
Other harmful aminoacidurias	9	50	10	20
Mucopoly saccharidoses, all forms	4	50	10	15
Galactosemia	1	5	1	2
Tay Sachs	1	10	2	1
Total recessive		*2,500*	*500*	*600*
Sex-linked recessives				
Haemophilia, both forms		100		
Duchennes dystrophy		200		
All others		200		
Total X-linked		*500*		
Autosomal dominants				
Epiloia		50		
Marfan's syndrome		50		
Achondroplasia		50		
Neurofibromatosis		50		
Dystrophia myotonia		50		
Blindness		100		
Deafness		100		
All others		150		
Total dominant		*600*		
Total genic disorders		*3,600*		

Note: Approximate incidences are based mainly on personal experience in Birmingham, using the accurately defined incidences as approximate standards. The individual components are thought to be correct to within a factor of 2, and their sum within a range of ±50 percent. Based on various sources, including Leck et al. 1968.

*NA refers to an approximate estimate of the equivalent number of alleles; for alleles of equal frequency, it is the number of these alleles.

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Table 2. Hereditary Disorders in Man: Chromosomal

Disorder	NA*	Incidence Birth	Incidence at 1 Month	Recurrent
Mongolism	21	1,300	1,200	25
Trisomy E	18	300	100	—
Trisomy D	13	200	50	—
Familial mongolism		20	20	5
Other familial		100	50	10
Missing chromosomes or parts		100	50	
Missing X chromosomes or long parts		200	200	
Severe sex chromosome defects		100	100	
Total severe		*2,200*	*1,600*	
XYY males		1,000	1,000	
XXY males		1,000	1,000	
XXX females		1,000	1,000	
XY females		100	100	
XX males		10	10	
Others		40	40	
Total mild		*3,200*	*3,200*	
Total mild and severe		*5,400*	*5,000*	

Note: Approximate incidences are based mainly on personal experience in Birmingham, using the accurately defined incidences as approximate standards. The individual components are thought to be correct to within a factor of 2, and their sum to within a range of ±50 percent.

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Paternity errors, which usually exceed 10^{-2}, will obscure effects expected with an incidence of 10^{-5} or less. Even maternity errors probably exceed 10^{-4} in countries in which births take place in hospitals or in nursing homes. If a new protein is found in a closed community, and there is no inconsistency in a child's phenotype compared with the parents', then good evidence of mutation exists. Most claims of unusual proteins not supported by extensive family evidence must be regarded as unsubstantiated. A rare exception is provided by the conspicuous dominant hemoglobinopathies, such as the methemoglobinopathies.

Even if based on many markers, good consistency in relation to paternity can rarely give an odds ratio of more than 10^3 to 1 for the apparent father, as opposed to a random father, and even at this level the odds for nonpaternity compete with those for a new protein. Eventually, positive evidence of paternity from very polymorphic systems capable of exact definitive by sequencing may overcome this. (Neel, 1970, 1971.)

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unity (i.e., no variants) were found in such a population, a minimal estimate of mutation rate including that for neutral variants would be available.

Since it is impossible, using present techniques, to estimate the mutation rate of any of the smaller units in man by direct observation on the consequences of mutation, we must look for guidance either from theoretical necessity or from analogy with other species or other systems.

Theoretical Arguments

Each generation arises from the reproduction of a subset of the total population. If there is no mutation (i.e., reproduction is exact), and if the population number is not increased by at least a factor of 2, then variability is necessarily lost since not all parental alleles can have their survival guaranteed. This loss of variability is most easily appreciated in the extreme case of a single variant at one locus which may or may not be included in the reproducing subset; if that variant is excluded, the variability is extinguished. However, reproduction is inexact because of mutation. In a steady state the loss of variation caused by sampling from a subset will be balanced by the gain from mutation. In essence, sampling will make the rarer type still rarer and mutation will make the rarer type commoner.

Kimura (1964, 1970) derives the steady-state solution as $n_e = 4N_e\mu + 1$, where n_e is the effective number of alleles, and N_e is the "variable effective" number of the population (see also Crow and Kimura, 1970). This defines the mutation rate necessary to maintain a variability if the mutants are devoid of selective value; alternately, we may regard it as an estimate of the "clearance" capacity of a breeding subset in the presence of neutral variants.

This imposes an upper limit on μ, which would be defined by a knowledge of N_e; this is capable of estimation from the variance of child-number, including zero, and n_e which is a matter of biochemical enumeration on population sampling. Unfortunately, it is of no immediate practical value in relation to the problems of mutation in man, for it relates, by definition, to "neutral" mutations, that is, to mutations without effect, and these, by definition, are harmless. A strong case has been made for the widespread occurrence of neutral mutations, such as those leading to protein variants which have no other consequence beyond showing unusual bands on electrophoresis or unusual spots on peptide digestion. But the case is unsatisfactory. If all mutants were without effect, the fact of variety need neither surprise nor alarm us. If mutants were usually deleterious, however, then neither our expectation nor our observations would make it possible to know it; for the chance of all loci having identical alleles is remote, since all individuals have an expectation of heterozygosity at thousands of loci.

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If mutations were neutral, this would account for their ubiquity; therefore they are neutral, therefore they are harmless. In fact, a correct interpretation would be that $k/(k + 1)$ mutants are harmless, therefore we can tolerate k times the mutation rate estimated on mutant proteins. There are no estimates based on mutant proteins.

Estimates from Other Species

All living organisms, excepting a few RNA viruses, have the basic genetic apparatus of a strand of DNA coded with four, or rarely five, repeating bases. As some organisms are very small and breed rapidly, up to 10^{10} individuals can be examined, and mutations in their hereditary apparatus can be studied with an economy and precision impossible in man. As there is no reason to suppose any mutagen acting at the nucleotide level should be species specific, there is no technical problem in using these organisms to screen for mutagens at the nucleotide level.

If the mutation rate of units longer than the genic sequences of nucleotides (e.g., small deficiencies) are to be defined, small mammals are usually used, in particular, mice. In mice, as in man, most of the mutations which have been studied are not understood in molecular terms, so that mutation rates for defined units are not available, and are biased by both commonness and conspicuousness.

Special Methods

Exposure of sperm, or their precursors, to X-rays increases the risk of embryonic damage, and scoring is fairly simple in mammals with numerous embryos and punctual or indiscreet mating habits. Severely damaged embryos are usually termed *dominant lethals*. It is not clear what the basis is for this lethality, although gross chromosomal imbalance is commonly observed. The term *dominant* is unfortunate and conforms neither to Mendel's use nor to its casual use in man as an abbreviation for "disorders manifest in heterozygotes."

In spite of some statements to the contrary (Epstein and Röhrborn, 1971), dominant lethals have no obvious relevance to dominant disorders in man, although they are an adequate justification for preventing the marketing of drugs which induce them.

The feasibility of human cell culture has provided an apparently perfect assay system for mutational studies. Cells can be grown, and their shape, movement, and microscopic appearance (including karyotype) can be studied. Further, they can be put into various media, and minority populations can be selected.

These methods, which will doubtlessly provide information on mutation rates for mitotic errors, whatever these are, cannot provide data on meiosis—

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which may be the major site of spontaneous mutations—and, as yet, cannot provide data on specific proteins in quantities adequate for sequencing. If, as is also possible, there are error-correcting mechanisms active in meiosis, as in the various master-slave models (for discussion see Whitehouse, 1973), mitotic estimates may exaggerate the rate of transmission of mutants.

If, as seems possible (Callan, 1967), chromosome structure is such that the normal *rearrangement mutants*, or chiasmata, which number about 50 per generation in both man and woman, are restricted to intergenic regions, then no particular proneness to intragenic mutants need be anticipated at meiosis. Where a paternal effect has been observed, as in achondroplasia (Penrose, 1955), Apert's syndrome (Blank, 1960), and Marfan's syndrome (Murdock et al., 1972), there seems to be substantial evidence to incriminate meiosis; however, the conditions are hardly likely to be "genic."

CONCLUSIONS

The present position on mutation rates can very roughly be summarized in man as in Table 3. At all levels in the genetic hierarchy the mutation rate per unit tends to be inversely proportional to the number of units. The numerical values are very approximate, and the attempt to partition them into likely times is based on poorly understood mechanisms. Until studies of mutation rate are made at the level of each element in the table, only limited advances in knowledge can be expected.

In the absence of any good estimates, it is difficult to offer informed advice on the hazards of increasing the mutation rate by a tolerance of environmental mutagens. Unfortunately, the limitations of the present estimates are rarely appreciated, and could hardly be inferred from the statements of international committees. It is usually assumed, on the basis of an implicit small-gene-number model, that if we can tolerate our present mutation rate (μ_n), then we can tolerate an artificial mutation rate (μ_a) if μ_a/μ_n is less than some arbitrary level, usually unity.

This argument assumes that our "load of mutations" can be increased with no worse consequence than a proportional increase in the cost of supporting it, and of carrying it from generation to generation. Unfortunately, on a large-gene-number model, the analogy with load or burden extends to the likelihood that, as with beasts of burden or aircraft, an increase in load may rapidly become insupportable so that a case for the arbitrary dependence of tolerance of artificial rates on natural rates might more realistically be based on some such criterion as $\mu_n + \mu_a <$ some value; any evidence for a high natural mutation rate might then be seen as an indication for extreme caution in increasing it.

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In the absence of any good estimates, it is difficult to offer informed advice on the hazards of increasing the mutation rate by a tolerance of environmental mutagens. Unfortunately, the limitations of the present estimates are rarely appreciated, and could hardly be inferred from the statements of international committees. It is usually assumed, on the basis of an implicit small-gene-number model, that if we can tolerate our present mutation rate (μ_n), then we can tolerate an artificial mutation rate (μ_a) if μ_a/μ_n is less than some arbitrary level, usually unity.

This argument assumes that our "load of mutations" can be increased with no worse consequence than a proportional increase in the cost of supporting it, and of carrying it from generation to generation. Unfortunately, on a large-gene-number model, the analogy with load or burden extends to the likelihood that, as with beasts of burden or aircraft, an increase in load may rapidly become insupportable so that a case for the arbitrary dependence of tolerance of artificial rates on natural rates might more realistically be based on some such criterion as $\mu_n + \mu_a <$ some value; any evidence for a high natural mutation rate might then be seen as an indication for extreme caution in increasing it.

A further hazard is that, once the mutational load is substantially

increased, a population may be unable to reduce the load even after the mutational agents are withdrawn, in which case that population would die out. The usual mechanism for the clearance of mutants is by a reduction in the fertility of the more burdened subjects; this depends on sufficient variance in burden and in fertility, and on a negative correlation between the load carried by persons and their potential for off-loading it onto their descendents. Populations who can tolerate a mutation rate with one breeding structure may be unable to tolerate it with another, especially if the variance in effective fertility is reduced.

Any increase in load will reduce the variance in load itself; it is easy to see, on a simple nonhierarchical model, that, if the standard error of sampling of n defective elements among many is $\sqrt{n}$, then the coefficient of variation will be $1/\sqrt{n}$; it is on this coefficient that selection must "bite."

Such a "load" can only be "carried" by a population if, and only if, the variance in the numbers of deleterious elements per individual, the variance in fertility of individuals, and the negative correlations of viability and effective fertility are sufficient. At present, changes in fertility pattern (and, to a lesser extent, in therapy) are reducing all three parameters, whereas the mutation rate is likely to increase, and in the long term, the reduction in phenotypic variance caused by outbreeding following improvements in transport and the deaths of languages, dialects, and local customs, is creating a breeding structure within which mutants can anticipate an even longer interval between their birth and their death.

Tolerance of an increase in mutational load cannot be assumed, and a knowledge of the natural mutation rate, however desirable for its own sake, cannot be used to justify any increase in artificial mutagenesis considering the present state of knowledge of the natural mutants and of the mechanisms available for their extinction.

Table 3. Mutational Events in Man Based on Present Estimates

Genetic Element (*Ratio*)	Approximate Mutation Rate	State of Cell at Mutational Event			
		Interphase	Mitosis	Meiosis	Zygote
Diploid set (x *2*)	10^{-2}	–	+	?	?
Haploid set (x *20*)	5×10^{-3}	–	–	+ (or)	+
Chromosome (x *2*)	5×10^{-3}	–	+	+	?
Chromosome arm (x *?*)	10^{-4}	(+)	–	+	–
Functional unit? (x *?*)	?	(+)	–	(+)	–
Repeated unit? (x *?*)	?	(+)	–	(+)	–
Genic unit (x *1000*)	10^{-6}*	+	–	?	–
Nucleotide	10^{-9}*	+	–	–	–

*per unit

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INTRODUCTION 18
CERTAIN SPECIFIC ENZYME VARIANTS OF MEDICAL GENETIC INTEREST 20
Nature of Genetic Defect in Metabolic Diseases 20
Variation in Enzymes Which Metabolize Chemical Carcinogens 23
Relationship to Carcinogenesis 24
Genetic Studies in Animals 25
Human Studies 25
CELLULAR ORIGINS OF NORMAL AND NEOPLASTIC TISSUES 26
Cell Fusion 27
In Normal Tissues in Vivo 27
Of Normal Cells in Vitro 28
In Tumor Development 29
Possible Clonal Origin of Tissues 30
Normal Tissues 30
Malignant Tissues 31
GENE EXPRESSION IN DEVELOPMENT 33
The Time of Embryonic Nuclear Gene Expression 34
Asynchronous Expression of Maternal Alleles 37
GENE ACTION IN TUMORS HAVING A HEREDITARY BASIS 41
EPILOGUE 43
REFERENCES 46

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Cell Fusion 27
In Normal Tissues in Vivo 27
Of Normal Cells in Vitro 28
In Tumor Development 29
Possible Clonal Origin of Tissues 30
Normal Tissues 30
Malignant Tissues 31
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INTRODUCTION

The term *biochemical polymorphism* refers to a genetic difference which is detectable by chemical means. Since most of the known gene products are proteins, the detection of these biochemical variations involves mainly a determination of protein differences. The protein variations are expressed in two general ways which are exploited by the biochemical geneticist: (1) alteration in enzyme activity, and (2) alteration in net charge of enzyme molecules.

Detection of altered enzyme activity uses for the most part classical quantitative enzyme techniques. Many of the biochemical polymorphisms involve diminution or loss in enzyme activity, and many of these constitute the well-known hereditary metabolic diseases. Diminished activity is often detectable in the heterozygote. Variants in this class of polymorphism are relatively rare, probably because of the high selective disadvantage.

Variations which confer differences in net charge of the protein molecule are detected mainly by electrophoresis. The advent of the zymogram technique, which involves staining for specific enzyme activity directly in the electrophoretic medium, has resulted in a veritable explosion of knowledge about biochemical polymorphisms. The frequency of this class is relatively high for the converse reason that many, or perhaps most, do *not* carry significant selective disadvantage. The very technique of detection in this case requires that enzyme activity be retained, although of course not necessarily in full amount. Most of the polymorphisms discussed in this review are based on electrophoretic information.

The zymogram method is particularly advantageous in several respects. In a screening program, this method permits the classification of relatively large numbers of individuals by requiring only small amounts of unpurified biological material. It enables one to distinguish the products of individual loci and their variant alleles in cases in which multiple loci code for enzymes with similar biochemical specificity. A peculiar advantage of the zymogram method is that, in multimeric enzymes (which constitute a rather high percentage of the total), heterozygotes produce intermediately migrating, "hybrid" molecules resulting from random association of allelic subunits. Since the number of such intermediate molecules is a function of the number of polypeptide subunits making up the enzyme (Markert, 1963; Shaw, 1964), this has been a useful method in clarifying the quaternary structure of a variety of enzymes (e.g., D. A. Wright et al., 1972; Fig. 1) and in studies requiring information on activity of alleles within a single cell.

The title of this review chapter subsumes a huge body of information. This necessarily has required considerable restriction of the areas covered. We have decided to focus on subjects in which enzyme polymorphisms (variation caused by different alleles at a single locus), or allozymes, in

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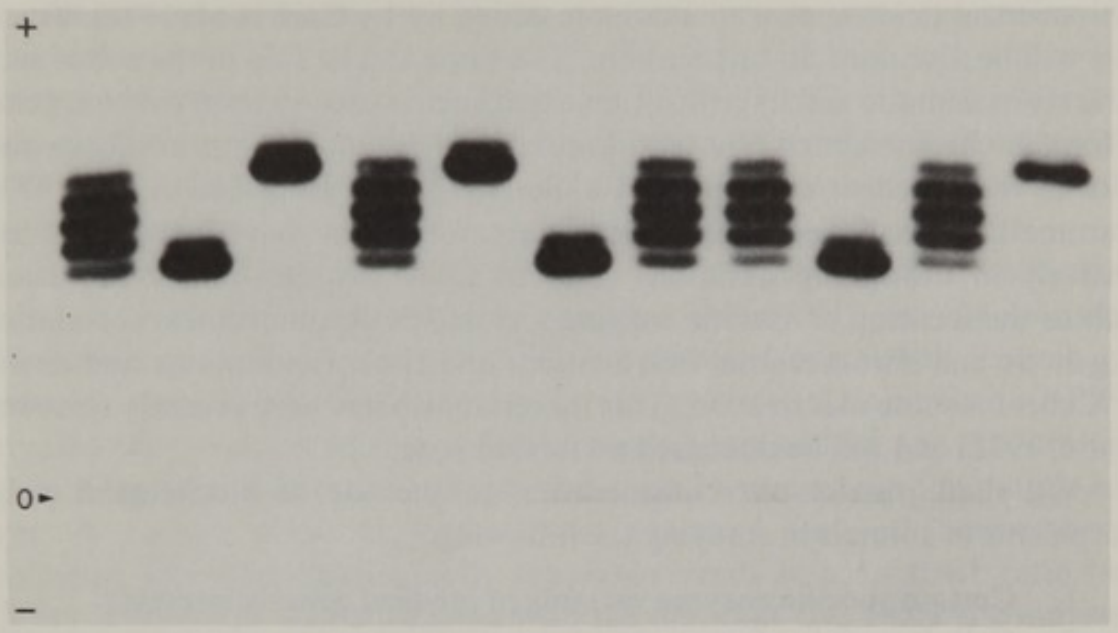
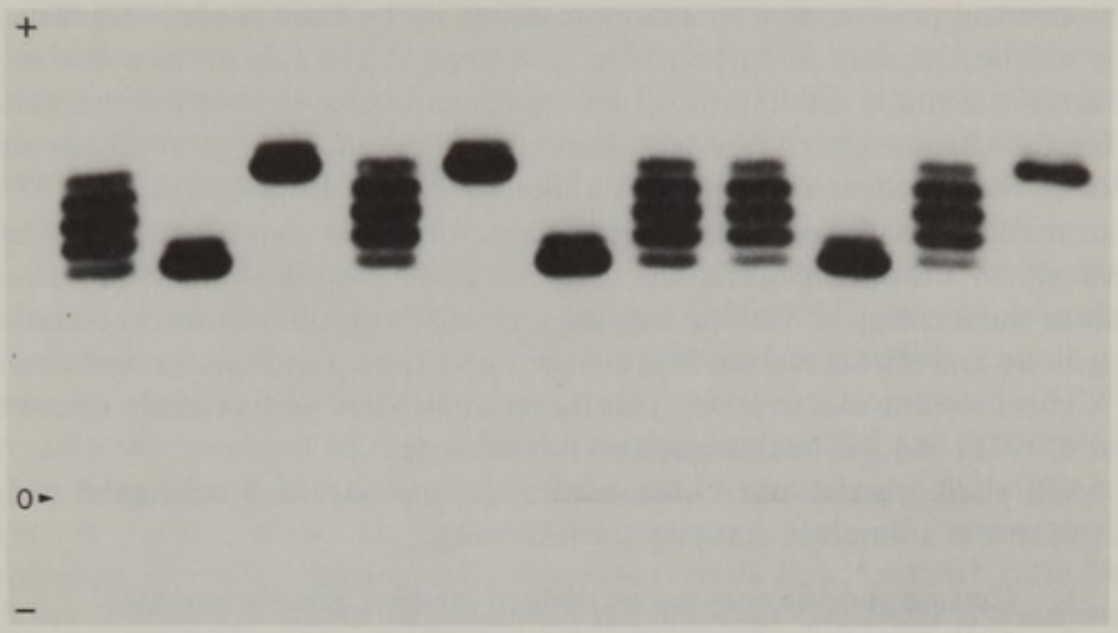


Fig. 1. Zymogram demonstrating phenotypes for the glyceraldehyde-3-dehydrogenase 1 (G-3-PDH-1) locus as seen in 11 F_2 hybrid fish after starch gel electrophoresis. Fish with multiple bands are heterozygotes containing one chromosome from each parental species. The five bands in these with relative activities of 1:4:6:4:1 indicate the tetramer structure of the enzyme. F_2 fish with single fast or slow migrating bands indicate homozygous fish in which one or the other of the parental chromosomes containing that locus have been recombined into the same organisms.

animals are being used to approach solutions to biological problems. Studies of methodology, enzyme polymorphisms in general, and considerations of molecular varieties of isozymes for their own sake are not rigorously considered: Appropriate review articles in these areas are available (Shaw, 1965; Shaw and Prasad, 1970; Shaw, 1969; Markert and Whitt, 1968).

An interesting and attractive area currently under investigation using zymogram methods in animal models is in determining and interpreting isozyme differences between normal and neoplastic tissues. However, these studies do not deal with allozymes but rather with the differential expression of genetic loci in which multiple loci code for the same type of enzyme. Ample, recent, comprehensive review articles have already been published covering the work suggesting the breakdown of enzyme control mechanisms, the activation of relatively nonspecific or embryonic enzyme loci, and the diminution of tissue specific isozymes in neoplastic cells (Criss, 1971; Weinhouse, 1972; Walker and Potter, 1972; Weinhouse et al., 1971). These topics will not be considered here.

The use of allozyme variants in animals in the study of population genetics and the impact of neutral alleles on molecular evolution are other large areas of investigation which have recently been reviewed (Harris, 1971; Gottlieb, 1971). A collection of papers by many of the leading figures in these



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We shall restrict our consideration to the use of biochemical polymorphisms in animals in studying the following:

1. Certain specific enzyme variants of medical genetic interest,
2. Cellular origin of normal and neoplastic tissues,
3. Gene expression in development,
4. Gene action in tumors having a hereditary basis.

CERTAIN SPECIFIC ENZYME VARIANTS OF MEDICAL GENETIC INTEREST

Studies on certain specific enzyme systems in our laboratory have stimulated our interest in two important problems related to them: the nature of the genetic defects in metabolic diseases, and the variation in enzymes which metabolize chemical carcinogens.

Nature of Genetic Defect in Metabolic Diseases

Many well-known hereditary metabolic diseases in man are the result of biochemical polymorphisms in which there is a great diminution or loss of enzyme activity (most of these are summarized in Harris, 1970b). It would be useful to know at what level in the progression of events from gene to enzyme activity such defects may manifest themselves. Consider the broad range of possible defects. A deletion resulting in the absence of the DNA coding for the enzyme would result in absence of m-RNA and the protein itself. A partial deletion or point mutation that could affect regions in the code and thereby disturb (1) transcription (with the resultant loss of mRNA and protein), (2) translation (so that mRNA was produced, but no protein), (3) secondary, tertiary, or quaternary structure of the enzyme producing mRNA (thus leading to protein of grossly anomalous structure and resultant loss of enzyme activity), or (4) an active site (with the production of mRNA and its translation into polypeptide subunits with unrestricted interaction, resulting in ostensibly normal protein molecules with the lack of enzyme activity).

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CERTAIN SPECIFIC ENZYME VARIANTS OF MEDICAL GENETIC INTEREST

Studies on certain specific enzyme systems in our laboratory have stimulated our interest in two important problems related to them: the nature of the genetic defects in metabolic diseases, and the variation in enzymes which metabolize chemical carcinogens.

Nature of Genetic Defect in Metabolic Diseases

Many well-known hereditary metabolic diseases in man are the result of biochemical polymorphisms in which there is a great diminution or loss of enzyme activity (most of these are summarized in Harris, 1970b). It would be useful to know at what level in the progression of events from gene to enzyme activity such defects may manifest themselves. Consider the broad range of possible defects. A deletion resulting in the absence of the DNA coding for the enzyme would result in absence of m-RNA and the protein itself. A partial deletion or point mutation that could affect regions in the code and thereby disturb (1) transcription (with the resultant loss of mRNA and protein), (2) translation (so that mRNA was produced, but no protein), (3) secondary, tertiary, or quaternary structure of the enzyme producing mRNA (thus leading to protein of grossly anomalous structure and resultant loss of enzyme activity), or (4) an active site (with the production of mRNA and its translation into polypeptide subunits with unrestricted interaction, resulting in ostensibly normal protein molecules with the lack of enzyme activity).

As pointed out by Harris (1970a), the resolution of these possibilities has been difficult for a variety of reasons. Enzyme proteins are not easily isolated and characterized from single individuals. They are usually detected and specified by their catalytic activity—the very attribute which is reduced or absent. It is often not possible to classify individuals unambiguously into discontinuous groups based on activity levels.

We have had an opportunity to study this question in allelic variants of glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) in the platyfish (*Xiphophorus maculatus*)—a popular viviparous tropical fish. Starch gel electrophoresis of individual tissues followed by in situ staining for G-3-PDH revealed the products of two different genetic loci coding for this protein. Locus 1 was active in brain and eye, and locus 2 was expressed in muscle and liver. A variant allele at locus 1 was observed with $\alpha\beta$ heterozygotes producing five electrophoretically separable bands in a 1:4:6:4:1 ratio (see Fig. 1). This result was consistent with the fact that G-3-PDH is composed of four polypeptide subunits (a tetramer) and that the product of the α allele combined randomly with the product of the β allele to produce, after electrophoresis of nervous tissue, the following 5 molecules in the above ratio: $\alpha\alpha\alpha\alpha$, $\alpha\alpha\alpha\beta$, $\alpha\alpha\beta\beta$, $\alpha\beta\beta\beta$, and $\beta\beta\beta\beta$ (D. A. Wright et al., 1972).

Only a common allele (α) was observed at locus 2 until a populational study revealed apparent heterozygotes ($\alpha\beta$) that produced a four-banded pattern after electrophoresis of liver and muscle tissue in the approximate ratio of 1:3:3:1 (see Fig. 2). This seemed to suggest the unlikely possibility that the α and β polypeptides produced by locus 2 combined to form not tetramer but trimer molecules: $1\alpha\alpha\alpha$, $3\alpha\alpha\beta$, $3\alpha\beta\beta$ and $1\beta\beta\beta$. An alternative hypothesis was that the variant allele (β) produced subunits which had little or no enzyme activity. These could have participated randomly with α subunits in tetramer formation, producing the 5 tetramer molecules, only 4 of which had visible activity. This would have produced four bands in a 1:3:3:1 ratio, as indicated in Table 1.

This latter hypothesis was supported by the following facts (Wright and Siciliano, 1973):

1. Crossing two heterozygotes produced four-banded heterozygotes to single-banded $\alpha\alpha$ homozygotes in a 2:1 ratio. Homozygotes for the variant allele ($\beta\beta$) were never generated; the lethality of this genotype is not surprising considering the importance of G-3-PDH in the glycolytic pathway.
2. Specific G-3-PDH activity in muscle and liver of heterozygotes was approximately half that of homozygotes.

This finding with G-3-PDH in fish is clearly supportive of the idea that some metabolic diseases may be caused by point mutations affecting only the active site of an enzyme molecule leaving other aspects of its production, three-dimensional structure, and interacting ability unaffected. Also, this

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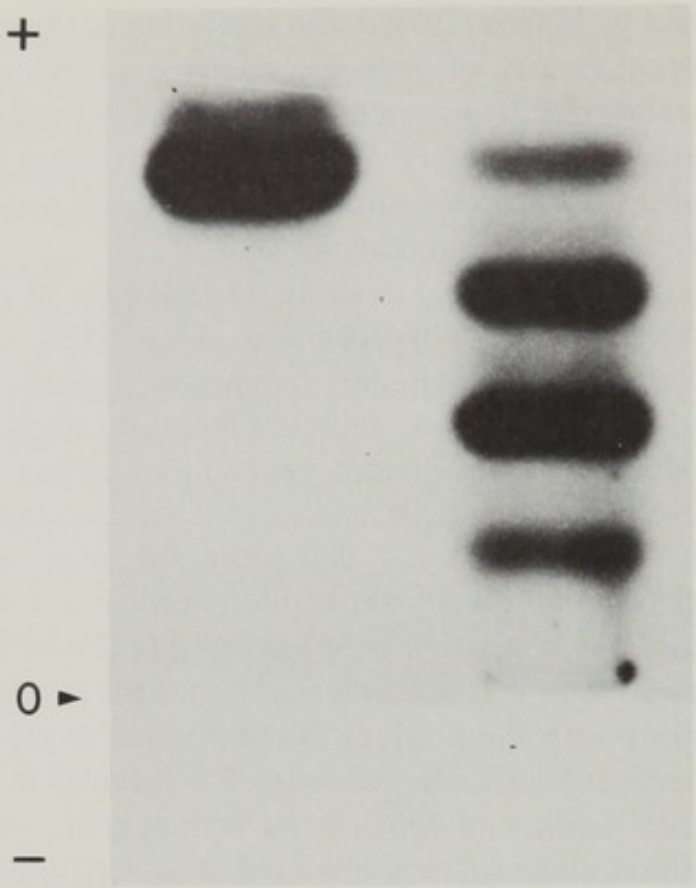
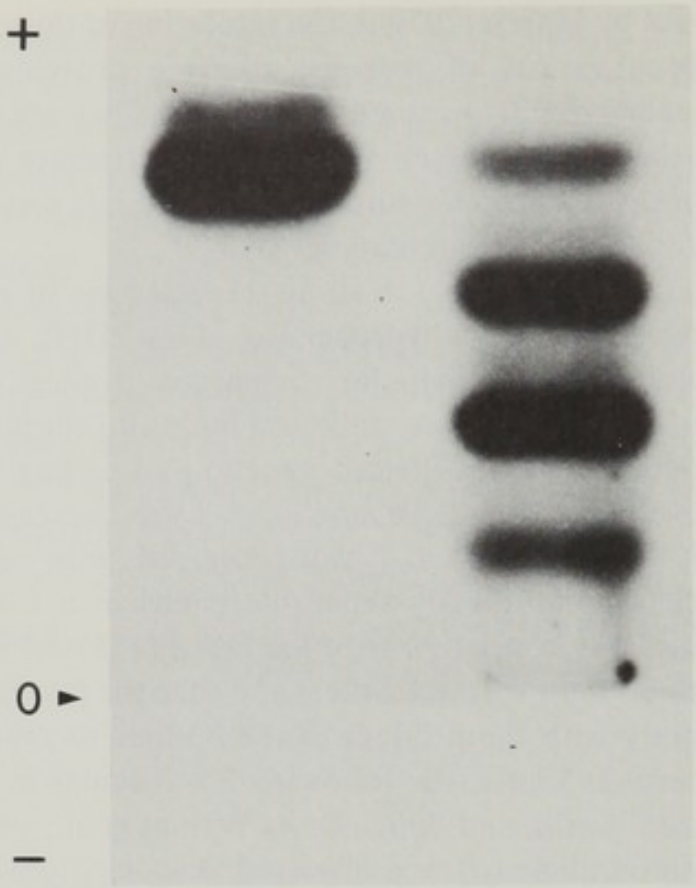


Fig. 2. Zymogram showing the products of the G-3-PDH-2 locus in fish of the genus *Xiphophorus*. Pattern at the left is typical of fish homozygous for the common allele. The four-banded pattern to right (with approximate relative activities of 1:3:3:1) is representative of a fish heterozygous for a new found variant allele.

Table 1. Activity of Bands after Electrophoresis of a Tetramer Enzyme Molecule from a Heterozygote in which the Product of a Null Allele Is a Subunit that Participates Randomly in Tetramer Formation with the Product of the Normal Allele Having a Different Electrophoretic Mobility

Band	Molecules Produced	Relative Amount of Each Molecule		Relative Activity of Each Molecule		Relative Activity of Each Band
1	$\alpha\alpha\alpha\alpha$	1	×	4/4	=	1
2	$\alpha\alpha\alpha\beta$	4	×	3/4	=	3
3	$\alpha\alpha\beta\beta$	6	×	2/4	=	3
4	$\alpha\beta\beta\beta$	4	×	1/4	=	1
5	$\beta\beta\beta\beta$	1	×	0/4	=	0



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4	$\alpha\beta\beta\beta$	4	×	1/4	=	1
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result provides a model for what can be expected as the result of electrophoresis of tissues from individuals heterozygous for such defects and as such could have an impact on genetic counseling. Some interesting questions of genetic regulation are also raised. Liver and muscle cells heterozygous at locus 2 and those homozygous for the variant lethal allele (which are never born) do not compensate for the resultant lower G-3-PDH activity by producing more α allele product (in heterozygotes) or any product of locus 1. Is this because otherwise normal protein is produced by the β allele at locus 2? Is it possible that vertebrate cells, unlike bacterial cells, do not have any enzyme regulatory mechanisms other than the turn-on of structural genes at the appropriate ontogenetic moment? Present data do not permit anything beyond speculation at this time, but a model system with which to study these problems is provided. Studies on the ontogeny of this enzyme system are now underway.

Variation in Enzymes Which Metabolize Chemical Carcinogens

A wide variety of chemical substances are known or suspected to have carcinogenic activity in experimental animals and man (see review by Miller, 1970). Probably the most important and widely occurring group of chemical carcinogens is the class known as *aromatic polycyclic hydrocarbons* (PCH).

Although the mechanism of action of the hydrocarbon carcinogens is not well understood, an early step in their action is the binding of the carcinogen to certain of the cell constituents, probably the DNA (Nebert and Bausserman, 1970; Grover and Sims, 1973). The hydrocarbons are metabolized after entry into the organism, and a major route of metabolism involves formation of the unstable and highly reactive epoxide. Recent evidence indicates that it is the epoxide form of the molecule which is the active carcinogen (Cookson et al., 1971; Huberman et al., 1972).

Metabolism of the hydrocarbon is illustrated in Figure 3. The initial step is enzymatic conversion of the native hydrocarbon to the epoxide by aryl hydrocarbon hydroxylase (AHH). The step to hydroxylation, forming the phenol, is spontaneous. Another important pathway of breakdown of the epoxide is by the enzyme epoxide hydrase (EH). The third pathway, by way of glutathione epoxide transferase, appears to be of less importance under most conditions.

The major enzymes in this metabolic transformation, AHH and EH, occur in the microsomal fraction of the cell, and are inducible by a variety of substances. Inducing agents include the usual hydrocarbon substrates, barbital, certain of the steroid hormones, and a wide variety of other unrelated compounds (see review by Conney, 1967). The highest activity of both enzymes occurs in the liver, but activity is distributed throughout many tissues of all mammals studied including man, and activity also occurs in cultured mammalian cells (Nebert and Gelboin, 1968; Kouri et al., 1972).

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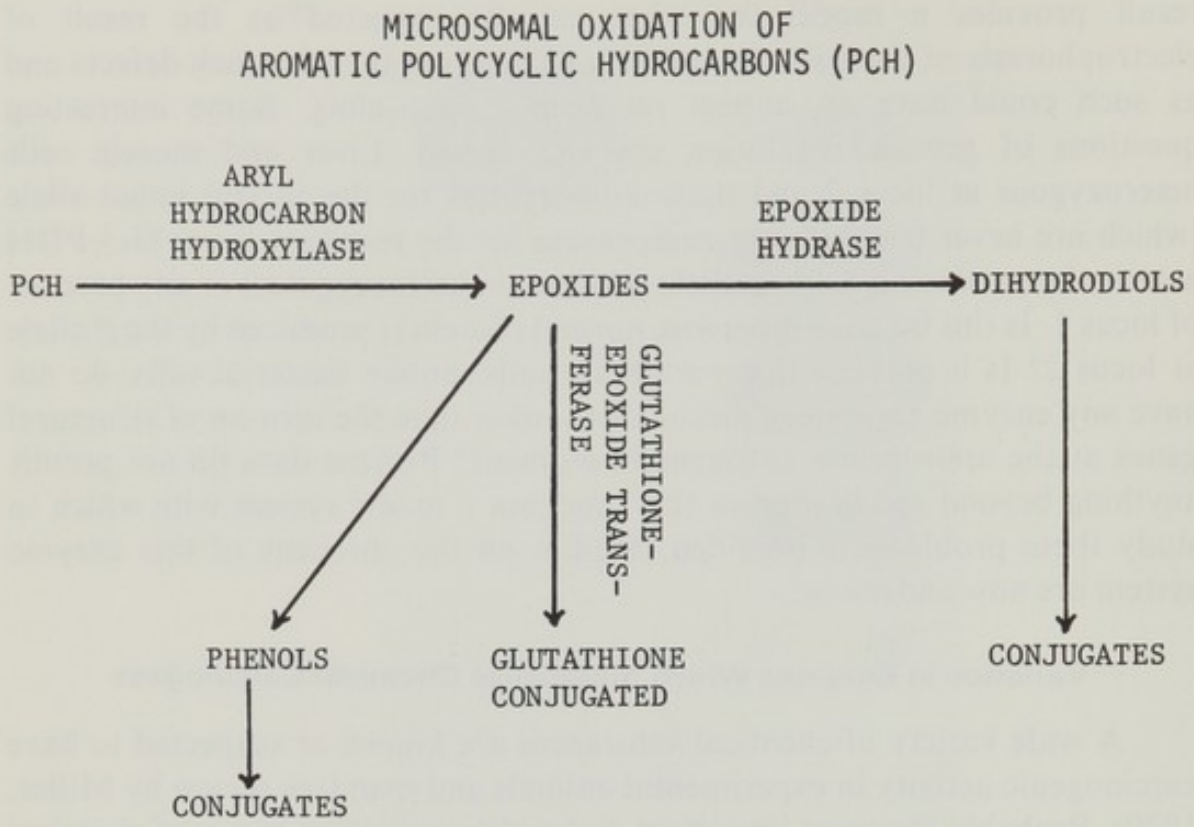
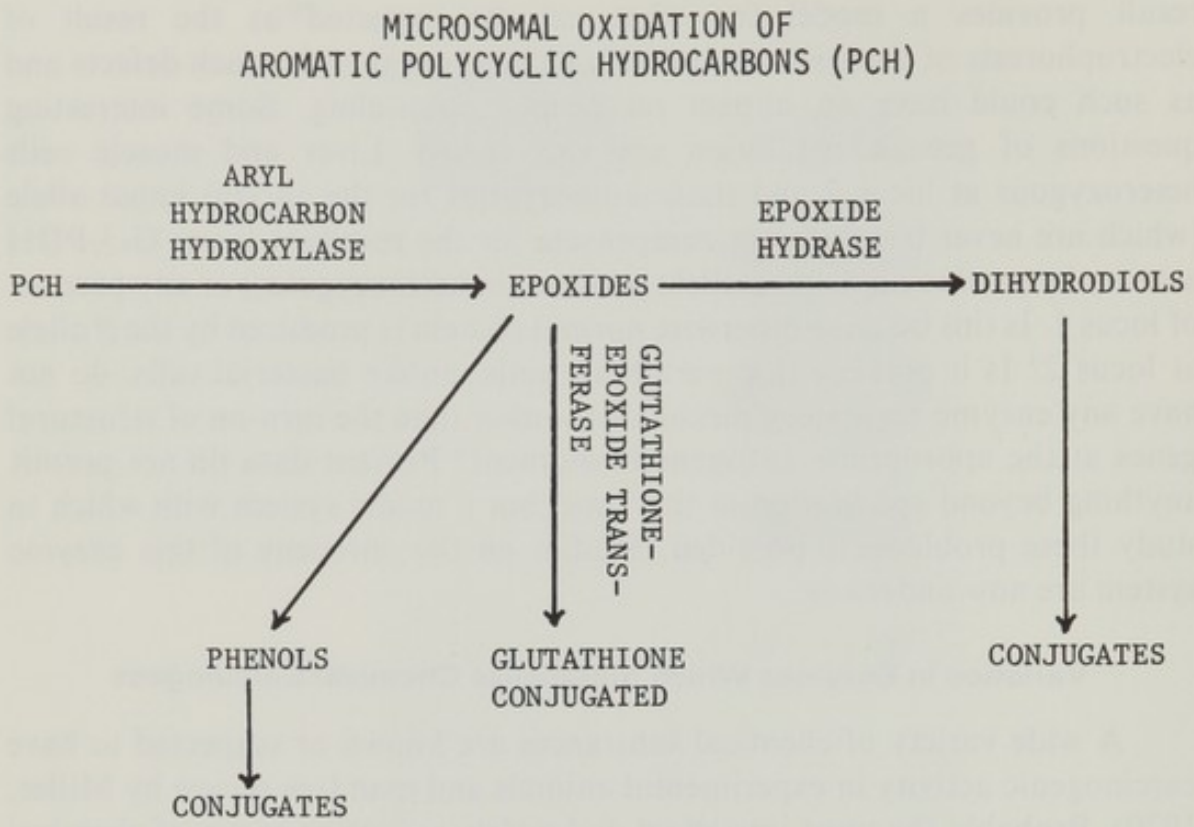


Fig. 3. Microsomal oxidation of aromatic polycyclic hydrocarbons (PCH).

Relationship To Carcinogenesis

Since AHH appears necessary for the formation of the active form of the hydrocarbon carcinogens, variation in the activity of the enzyme apparently plays a role in the susceptibility of the organism to chemical carcinogenesis (Gelboin and Wiebel, 1971; Gelboin et al., 1970; Gelboin, 1972). Although a number of animal experiments have been carried out on this subject, results are far from clear. Part of the confusion probably arises because the liver, in which there is the greatest activity, may act as a detoxifying organ and thus metabolize the carcinogenic compound to the phenol conjugate before it reaches the target organ. Also, the use of different strains of mice may have added to the confusion because of their multiple genetic differences. Thus, some studies indicate that animals with high AHH activity are less susceptible to certain experimental tumors. In this regard high inducibility of the enzyme has been considered to have a protective effect and has led to the suggestion (Wattenberg, 1966; Alfred et al., 1969) that the organism is safer from chemical carcinogenesis if given small amounts of inducing agents other than the carcinogen and thus maintains a high level of AHH.

On the other hand, high AHH activity can, under other conditions, increase susceptibility to chemically induced cancer (Gelboin and Wiebel, 1971). Further studies are required to clarify the apparent contradiction on this point.



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Genetic Studies In Animals

Strains of laboratory mice vary widely in their AHH activities both resting and induced. The differences are especially marked following administration of an inducing agent: for example, the DBA strain of mice did not show any increase in AHH activity after injection of 3-methylcholanthrene (3-MC), whereas the C57 BL/6 strain showed an increase of four and a half times above resting levels (Thomas and Hutton, 1973). Crosses between inducible and noninducible strains of mice indicate that control of the inducibility is by a single genetic locus (Thomas et al. 1972; Nebert and Gielen, 1972). The genetic differences in inducibility of enzymes were demonstrable in cells cultured from the different strains of mice as well as in the whole animal. Apparently, a second locus, probably a structural gene, is involved in some of the strain differences (Thomas and Hutton, 1973).

Epoxide hydrase has been less thoroughly studied both genetically and in its relationship to tumorigenicity. In a recent study (Nebert et al., 1972), no differences in hepatic EH between C57 BL/6 and DBA mice occurred.

Human Studies

Early efforts to extend the findings on these carcinogen-metabolizing enzymes to human subjects were hampered by difficulty in obtaining tissue samples from large numbers of individuals. Most of the animal studies were done on liver tissue, and required either killing the animal or taking a liver biopsy, a procedure not routinely feasible in man. Reports of small series of human subjects included studies of placental tissue, foreskins from newborn males, and cultured fibroblasts from skin biopsies (Nebert et al., 1969; Welch et al., 1968; Levin et al., 1972; Huberman and Sachs, 1973). These indicated variability in man, but the numbers were too small for statistical significance.

The need for an assay method on readily accessible human tissue led to the development of a technique using cultured human lymphocytes (Busbee et al., 1972). The cultured lymphocyte method was first developed for AHH, and was subsequently adapted to measurement of EH (Kellermann et al., 1973a). Both assays can now be carried out on 10-ml specimens of venous blood.

Using the cultured lymphocyte assay for AHH, a preliminary survey of approximately 100 normal, healthy volunteers demonstrated considerable variation in the extent of inducibility, from approximately one and a half to five times resting levels (Kellermann et al., 1973b). The study was then extended to include families. A total of 353 subjects were studied, including 67 families with 165 children. Based on the amount of inducibility, the subjects fell into three groups, arbitrarily called low, intermediate, and high. Amount of inducibility correlated well with resting levels (low inducers had low resting levels of activity, etc.). Assuming a single gene control, the

numbers in the three groups fit the Hardy-Weinberg equilibrium ratio, with the high and low groups representing the two homozygous groups and the intermediate group being the heterozygotes. Gene frequencies for the low and high activity genes were 0.717 and 0.283, respectively, Families representing all six possible crosses were obtained and in no case did the offspring deviate from expected results (Kellermann et al., 1973c).

To determine whether levels of activity in the cultured lymphocytes were representative of other tissues, pulmonary alveolar macrophages were obtained from several volunteers by bronchoscopy with saline alveolar lavage. In the 12 cases studied, levels of activity agreed closely in the two tissues. Studies are presently underway on fibroblasts cultured from skin biopsy.

The epoxide hydrase technique has only recently been developed and a total of 60 subjects have been studied. This enzyme is apparently less inducible than AHH, with levels arising to no more than two times resting levels. However, degree of inducibility of AHH and EH parallel—the correlation is virtually 100 percent. Thus, apparently the inducibility of the two enzymes is under the same genetic control (Kellermann, unpublished).

Of the chemically induced tumors in man, bronchogenic carcinoma is probably the most common. Studies are now in progress to determine if occurrence of this tumor shows any relationship to levels of activity of AHH and EH. Again, the number of subjects studied is too small for conclusive evaluation. Whatever these results, the animal studies demonstrating genetic variation in AHH inducibility led directly to the research in man, which has demonstrated that the same general finding holds for the human organism. Hopefully, this will pave the way for improved understanding of chemical carcinogenesis in man, and lead to screening methods for evaluating susceptibility to certain chemically induced tumors.

CELLULAR ORIGINS OF NORMAL AND NEOPLASTIC TISSUES

The principles of biochemical polymorphism have been applied to the solution of two questions that have been asked about the cellular origin of normal and neoplastic tissues both in vivo and in vitro: (1) To what extent does cell fusion and the resultant somatic transfer of genetic information play a role? (2) To what extent do all the cells of particular tissues or of a particular tumor originate from a single common cell or group of cells?

To study these questions in an animal model, two conditions must be met. First, the organisms or tissues under study need to be composed of at least two different genetic populations of cells. That is, at least some form of genetic mosaicism need exist. Second, there must be some means of detecting or recognizing the cells of different genetic constitution in the tissue or tumor

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under consideration. This is where the biochemical polymorphisms play a role.

Cell Fusion

In Normal Tissues In Vivo

In studying this and other problems related to the origin of normal and neoplastic tissues, it has proved most useful to exploit the mosaicism achieved by the manufacture of the allophenic mouse (Mintz, 1965). Such a mouse is created by taking two cleavage-stage embryos of different genotypes, dissociating the blastomeres, mixing them together so that they reaggregate into one embryo, and transferring the composite to an "incubator" mother for the completion of development. The mouse produced is therefore a mosaic composed of two genetically different types of cells.

Using this system and taking advantage of a biochemical polymorphism at the nicotinamide adenine dinucleotide phosphate (NADP)-dependent isocitrate dehydrogenase (IDH) locus, Mintz and Baker (1967) investigated the possibility of cell fusion in various tissues with particular reference to skeletal muscle. The parental strains used to produce the allophenics differed in the electrophoretic mobility of IDH, so that the cells from one of the embryos had a slower migrating form of the enzyme ($\alpha\alpha$) than the cells from the other ($\beta\beta$). Tissues composed of both kinds of cells would therefore have been expected to produce both bands. When both genomes are located in the same cell (after cell fusion), the polypeptide subunits from each message should be assembled randomly into dimer IDH molecules to produce three bands, the two original and the heteropolymer ($\alpha\beta$). The dimer structure of IDH and the resultant three-banded pattern in heterozygous cells has been well demonstrated in a variety of animals (Henderson, 1965; Siciliano and Wright, 1973a).

With these tools, Mintz and Baker (1967) therefore set out to settle a classical argument: Are the multinucleated fibers of syncytial skeletal muscle tissue produced by the fusion of myoblasts or by nuclear division not followed by cytokinesis? If the latter, any one fiber, though multinucleate, would contain only one genome and produce one or the other parental IDH band, so that muscle composed of many fibers should have two bands. If fusion had taken place in the allophenics, some (indeed most) of the fusions should have been between cells of different IDH genotypes, so that fibers would contain both genomes and assemble IDH from two different polypeptides producing the three species of molecules (three bands after electrophoresis) as in a hybrid cell. The three-banded pattern was present in the skeletal muscle of allophenic mice, conclusively demonstrating myoblast fusion in vivo in the production of this syncytium. Similar results were later obtained making use of NADP-dependent malate dehydrogenase (MDH) polymorphisms (Baker and Mintz, 1969). Two-banded patterns for IDH only were

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With these tools, Mintz and Baker (1967) therefore set out to settle a classical argument: Are the multinucleated fibers of syncytial skeletal muscle tissue produced by the fusion of myoblasts or by nuclear division not followed by cytokinesis? If the latter, any one fiber, though multinucleate, would contain only one genome and produce one or the other parental IDH band, so that muscle composed of many fibers should have two bands. If fusion had taken place in the allophenics, some (indeed most) of the fusions should have been between cells of different IDH genotypes, so that fibers would contain both genomes and assemble IDH from two different polypeptides producing the three species of molecules (three bands after electrophoresis) as in a hybrid cell. The three-banded pattern was present in the skeletal muscle of allophenic mice, conclusively demonstrating myoblast fusion in vivo in the production of this syncytium. Similar results were later obtained making use of NADP-dependent malate dehydrogenase (MDH) polymorphisms (Baker and Mintz, 1969). Two-banded patterns for IDH only were

detected in extracts from liver, kidney, lung, and spleen of the same animals. The absence of hybrid enzyme in these tissues suggests that cell fusion is not a common phenomenon in them, if it occurs at all. Interestingly, the hybrid enzyme was also missing in cardiac muscle (Mintz and Baker, 1967), which superficially resembles a syncytium. This leaves open the question as to whether cardiac muscle is a true syncytium and suggests that, if it is, perhaps it is formed, contrary to skeletal muscle, by karyokinesis in the absence of cytokinesis (endoreduplication).

A similar experiment was conducted to examine the possibility of fusion in the apparent multinucleated syncytium of the placental trophoblast (Gearhart and Mintz, 1972b; Chapman et al., 1972). Here, an electrophoretic polymorphism at a glucose phosphate isomerase (GPI) locus was used. Carter and Parr (1967) showed that GPI was a dimer which produced a hybrid band in heterozygous cells. To look for fusion between maternal-fetal trophoblast cells, embryos of mice of one GPI genotype were implanted into the uterus of incubator mothers of a different genotype. To look for fetal-fetal fusion, allophenics (or *chimeras* as Chapman et al. refer to them) were manufactured from embryos of contrasting GPI genotypes and implanted into an incubator uterus of a mother with one of the genotypes. In either case, presence of the hybrid enzyme would have been indicative of fusion, but none occurred. Therefore, apparently the giant cells of the mouse trophoblast arose by phagocytosis, or by formation of a syncytium by endoreduplication, or by both. Thus, skeletal muscle is the only normal tissue known to be formed in vivo by cell fusion.

In Normal Cells In Vitro

In vitro fusion of somatic cells (Barski and Cornefort, 1962) and animal biochemical polymorphisms used therein have been most helpful in mapping human chromosomes in cases in which human cells have been fused with animal cells. As human chromosomes are lost (a form of segregation) from cells of various hybrid clones (Weiss and Green, 1967), the concordant segregation of human enzyme loci (indicated by loss of human enzyme from such clones) gives clues to the synteny (linkage) of these loci. As mentioned above, these linkage experiments have been comprehensively reviewed recently and will not be discussed further here (Ruddle, 1972). However, a recent question regarding this system has been neatly answered using animal biochemical polymorphisms. In fusing man and mouse cells, multiplicities greater than 1:1 (one human to one mouse genome) have been reported (Matsuya et al., 1968; Nabholz et al., 1969). The question is can the multiplicity greater than 1:1 be due to the fusion of more than two cells or due to fusion of only two cells followed by an endoreduplication of only one set (probably mouse) of chromosomes? Evidence for the former has been presented by Ricciuti and Ruddle (1971). They used three different cell lines

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in a fusion experiment (one human and two mouse), each containing a different electrophoretic variant for GPI. They then isolated a clone of cells with a model chromosome count indicating a multiplicity >1:1. These same cells produced all three forms of GPI, indicating that more than two cells can fuse to form a viable hybrid.

In Tumor Development

The above information taken together with the numerous examples of hyperdiploidy in cells of neoplastic tissues leads one to ask whether cell fusion may be involved in tumor development. Goldenberg et al. (1971) took cells derived from a human lymphoma and grew them in a hamster cheek pouch. They were serially transmitted from hamster to hamster and caused severe malignancy and metastasis. Tissue culture cells derived from these transplantable tumors were tested for the presence of human and hamster forms of lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G-6-PDH) by electrophoresis. Only hamster type LDH patterns were observed in all cell lines tested, but 8 of 24 cell lines demonstrated both hamster and human type (as in HeLa cells) G-6-PDH. The authors conclude that it is likely that the human tumor and hamster host cells fused and that most of the human chromosomes were lost (eliminating histocompatibility problems). They conclude further that the retention of at least part of the human X chromosome is the reason for the human G-6-PDH activity in the cells studied. They further entertain the hypothesis that in vivo somatic cell fusion might be an important mechanism in the evolution and progression of tumors to their ultimate state of malignancy.

As interesting as this hypothesis may be, there are some serious difficulties with this work which cast doubt over the conclusions, to say nothing of the hypothesis. If fusion had taken place, one would expect hybrid molecules between the hamster and human G-6-PDH (Boone and Ruddle, 1969). None appeared. In addition, the human G-6-PDH seen appeared to be the product of the A allele variant common in HeLa cells. This particular variant has occurred only in Negroes (from which the HeLa line was derived, Gartler, 1968). The patient from whom the human tumor cells (neither patient nor original cells were any longer available for study) were derived was Caucasian. These facts suggest HeLa cell contamination of the transplantable tumor sublines (a frequent occurrence in many laboratories, according to Gartler, 1968) rather than their being the result of cell fusion. Goldenberg et al. (1971), aware of this interpretation, countered by noting the absence of human type LDH patterns in the cell lines studied and suggested that the A type electrophoretic variant of G-6-PDH in many spontaneous human cell lines could arise by point mutation (a contention supported by Steele, 1970). However, the electrophoretic separation on the gel systems used in this work was such that the human versus hamster LDH

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patterns were not unambiguously resolved. The point mutation suggestion apparently is not consistent with the large number of reports, many summarized in this paper, in which the validity of allozyme markers in indicating the origin of cells has been well demonstrated, nor with the authors' own attempt to relate the presence of human G-6-PDH to a cell fusion event. At any rate, the paper under discussion does demonstrate the limitations imposed and minimum precautions that one must take to use these markers effectively: gel work with clear resolution of allozyme variants, use of original or parental genomes as controls.

The question of cell fusion in the evolution of malignancy is still open. However, using the allophenic mouse system, Mintz (1970) reported mosaic tumors (see next section) in which no hybrid enzyme molecules were detected, suggesting that cell fusion and other forms of genetic exchange are not factors involved with neoplastic transformation in vivo.

Possible Clonal Origin of Tissues

Normal Tissues

Using allophenic mouse embryos made up of cells with different electrophoretic types of GPI, Gearhart and Mintz (1972a) investigated the possible clonal origin of individual somites and their muscle derivatives. In most cases, both electrophoretic types of GPI were detected in a single somite, indicating that each arose from at least more than one cell (nonclonal). The proportion of cell strains making up each somite was most likely reflected in the proportion of the amounts of the two different types of GPI in the somite. Assuming this, they noted that although proportions of the two-cell strains varied among the somites of an embryo, small groupings of two-to-three sequential somites seemed to have similar genotype proportions. This seemed to indicate that although somites did not have a clonal origin, small groups of somites likely shared common origin from the same group of cells.

Each individual eye muscle examined in this study (each formed from the myotome region of a single somite) also contained both GPIs. So, even a portion (approximately one-third) of a somite still originated from at least two cells. In fact, more than two cells are indicated since both forms of GPI were in all 33 eye muscles studied. If just two cells were involved in giving rise to the structure, 50 percent of the time one would expect both cells to be of the same strain producing only one form of GPI. Presence of both forms in all 33 muscles suggests the myotome arose from at least five cells.

Gearhart and Mintz (1972a) presented this information on mosaics as a model to explain the wide range of phenotypes in females heterozygous for X-linked genetic diseases (e.g., Duchenne-type muscular dystrophy). In these

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cases, because of random X-chromosome inactivation, heterozygotes are phenotypic mosaics. Therefore the severity of the disease in an affected female depends on the proportion of cells in which the X bearing the normal gene is inactivated.

Using GPI variants in allophenic mice, Mintz (1971) looked at the proportion of the two-cell strains (once again by observing the proportion of the two different electrophoretic variants) in red versus white blood cells. There was a high correlation of genotypic proportions of the two-cell strains in red and white blood cells of the same individuals despite an unrelatedness of these proportions in other tissues. Red and white blood cells were therefore seen arising from a common pool of precursor cells. This result correlated well with the results of Gandini and Gartler (1969) in man. In this case, females heterozygous for electrophoretic variants of G-6-PDH were used. Since this is a sex-linked trait, the females were mosaic for the phenotype. They found a perfect correlation for the amount of one variant to the other in red blood cells, granulocytes, and lymphocytes from the same individual; this was compatible with the model of common precursor or totipotent stem cells for these three cell types.

Mintz (1971) found that one liver lobe of an allophenic mouse contained only one form of NADP-MDH (composed of only one strain of cells) and the other lobe expressed both homopolymers, indicating that each lobe originated from separate groups or clones and that a single lobe arose from at least two clones. This was verified and the information extended by Condamine et al. (1971), once again working with the allophenic mouse system. Here the biochemical polymorphism used was high and low β-glucouronidase activity. Histochemical staining followed by microscopy of allophenic livers revealed clusters of cells in the same liver lobe. Some clusters had high, others low enzyme activity, once again indicating the multiclonal origin of cells of the same liver lobe.

Malignant Tissues

The possible clonal origin of malignant cells in a tumor has been considered an interesting and important question. It would indicate, if true, that cancer was the result of a rare event happening in only a single cell which proliferated abnormally leading to tumor and metastasis. The above methods using biochemical polymorphisms in mosaic animals exploited in analyzing the origin of normal tissues have been applied to tumor analysis. The allophenic mouse, though providing some valuable and interesting information on tumor biology, has been of only limited value in this question (for summary, see Mintz, 1970; Mintz, et al., 1971). In these cases, allophenics were constructed between high- and low-incidence strains for various kinds of tumors (mammary, liver, lung, leukemia). Tumors produced in the allophenics (usually specific to the high tumor line) were analyzed for

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mosaicism (presence of both parental allozymes). Usually only one allozyme was present, suggesting the clonal origin of tumor cells. However, since the single allozyme present was always consistent with the characteristics of the tumor typical of the strain represented by the allozyme (frequency, age of onset, number of tumors, growth characteristics), the results suggested only that the cells of one strain participated in tumor formation and do not offer any information to eliminate the possibility that more than one cell of that strain was initially involved. An exception to these results was noted in hepatomas produced in allophenic mice. In one study (Mintz, 1970), of 23 hepatomas examined in allophenic mice for NADP dependent MDH, two were mosaic having mostly MDH from one strain with a minor component of MDH from the other. Whether the cells responsible for the minor component were tumorous or just normal cells mixed in with or trapped by the main body of the malignancy could not be ascertained. In another study (Condamine et al., 1971), 12 hepatomas in allophenic mice were examined histochemically for high and low levels of β-glucouronidase (which indicated the strain of origin of the cells). In two such tumors malignant cells for both genotypes were seen. This latter evidence suggests, therefore, that carcinogenesis may involve some measure of cell interaction (though not fusion as indicated by the lack of hybrid bands in mosaic tumors) or intercellular transmission of tumor information, and that there may be independent malignant transformation in two or more cells.

Better resolution of these alternatives should be possible in systems in which the variants responsible for the biochemical mosaicism are not necessarily related to potential for malignancy. Happily, this situation presents itself in humans. As indicated above, females heterozygous for electrophoretic variants of G-6-PDH will have one or the other gene expressed, depending on which X chromosome is inactivated, in different cells. Consequently, the presence of both electrophoretic forms in tumors of heterozygous females would be consistent with multicellular origin, whereas the presence of only a single form would indicate the clonal origin of tumors.

As in the allophenic mouse work, both types of results have been obtained. Only one form of G-6-PDH was found in leiomyomas of heterozygous females, whereas both forms were present in muscle tissue adjacent to the tumors (Linder and Gartler, 1965). However, both enzyme types were seen in hereditary trichoepithelioma (Gartler et al., 1966). Summarizing similar experiments conducted by himself and others, Fialkow (1970) also noted from different tumors results consistent with both concepts—unicellular and multicellular origin of neoplastic tissues. Aside from the trivial explanations for the different results (e.g., normal cells of different genotype mixed in with tumor cells giving a mosaic pattern), he did point out that the differences in the results might have relevance to the etiology (mostly unknown) of the tumor under study: genetic, viral, radiation induced,

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immunological. Such a consideration should be of importance in studying cancers with a known or suspected genetic etiology. Since, in such cases, cells are already predisposed to cancer, one would expect a greater likelihood of more than one cell being transformed to the malignant state than one would expect in the nongenetic situation (Knudson, 1971). Surely, this would be consistent with the observation of multiple primary lesions being part of the syndrome for genetic cancers (see summary of Knudson et al., 1973) and with both types of G-6-PDH seen in hereditary trichoepithelioma (Gartler et al., 1966). Studying cancers with etiology in mind and making use of electrophoretic methods for newly discovered sex-linked enzyme loci (e.g., 6-phosphogluconate kinase, Chen et al., 1971; Meera Khan et al., 1971) may therefore have some considerable impact on uncovering cancers with a genetic basis and on the resultant identification of individuals at high risk, as well as on early diagnosis and prevention of cancer in man.

Smith et al. (1971) suggested quite a different interpretation for mosaic carcinomas of the cervix in Negro females heterozygous for G-6-PDH. All six patients with preinvasive cervical neoplasia displayed only one G-6-PDH band consistent with clonal origin. However, two of seven patients with invasive carcinoma of the cervix, in which histologic examination revealed no contaminating normal tissue, displayed the typical mosaic pattern—two bands or the products of both alleles. Since hybrid bands of G-6-PDH did not appear, the authors suggested that the phenomenon did not represent a breakdown of X-chromosome inactivation in cancer cells and that both X chromosomes were not active in the same cell. Multiclonal origin of the cancer cells was indicated in the invasive carcinoma. They hypothesized that the uni- or multiclonal situation was a function of the extent of the neoplasia—preinvasive or invasive. McCurdy (1967) arrived at a similar conclusion after studying a variety of metastatic and nonmetastatic tumors. Clearly, there is a need for further study of this phenomenon, perhaps in using sex-linked polymorphisms in different etiological types of tumors from mammalian animal models, in which experimental manipulation can be performed and larger numbers generated.

GENE EXPRESSION IN DEVELOPMENT

Many enzymes, especially those associated with general metabolism common to all cells, are found in unfertilized eggs. The phenotype of such an enzyme would be controlled by the maternal genome. In subsequent development of the fertilized egg, enzyme activities might increase. An increase in enzyme activity might reflect new synthesis due to activation of genes in the maternal and paternal chromosomes, new synthesis of enzyme directed by maternal messenger ribonucleic acid (RNA) or simply activation

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of previously inactive preformed enzyme molecules. Use of genetic variants allows us to examine the time of expression of the zygote's genome evidenced by the appearance of a hybrid enzyme pattern containing products of both maternal and paternal alleles.

With these kinds of expectations, we can examine the expression of enzyme patterns in embryos heterozygous for various enzyme loci. Two points can be considered: (1) the time of embryonic nuclear gene expression, and (2) asynchronous expression of maternal alleles in development.

The Time of Embryonic Nuclear Gene Expression

The ideal type of system to determine when a genetic locus for a particular enzyme is expressed would be a system in which the only genetic differences between the parents were at that locus, so that any regulatory mechanisms would affect each allele identically. These conditions are approximated in intraspecific crosses, especially when genetically defined strains are used.

Crosses using animals of closely related or the same species, but from different populations or subspecies, provide a rich source of electrophoretic variants but may introduce some complex genetic interactions into the development of the hybrids. Differential effects on allelic expression can be tested in such hybrids, however, by making reciprocal hybrids. If the results are the same, one can assume that the regulatory factors are the same in both subpopulations of the species.

Perhaps the best understood genetic system in metazoans is *Drosophila melanogaster,* in which there are a great many biochemical polymorphisms (D. A. Wright and Shaw, 1970; Ursprung et al., 1968; Ursprung and Leone, 1965; Dickinson, 1968; Madhavian et al., 1972). A complete review has recently been published by T. Wright (1970).

Several enzymes have been studied in Drosophila for the appearance of paternal gene expression. Strains of Drosophila having different electrophoretic forms of enzymes are crossed in mass matings. The embryos up until 18 to 20 hr of development at 24 C have only the maternal form of IDH, α-glycerophosphate dehydrogenase (αGPDH), G-6-PDH, 6-phosphogluconate dehydrogenase (6-PGD) and alcohol dehydrogenase (ADH). After 18 to 20 hr, hybrid (heterozygous) enzyme patterns for each enzyme are observed. The expression of the heterozygous enzyme pattern corresponds to an increase in total enzyme activity for each enzyme (Wright and Shaw, 1969, 1970). In newly hatched heterozygous embryos, the maternal form is more active than the paternal form. Interpretation of this maternal effect is made difficult because maternal nuclear and cytoplasmic contributions to the phenotype are not distinguished.

of previously inactive preformed enzyme molecules. Use of genetic variants allows us to examine the time of expression of the zygote's genome evidenced by the appearance of a hybrid enzyme pattern containing products of both maternal and paternal alleles.

With these kinds of expectations, we can examine the expression of enzyme patterns in embryos heterozygous for various enzyme loci. Two points can be considered: (1) the time of embryonic nuclear gene expression, and (2) asynchronous expression of maternal alleles in development.

The Time of Embryonic Nuclear Gene Expression

The ideal type of system to determine when a genetic locus for a particular enzyme is expressed would be a system in which the only genetic differences between the parents were at that locus, so that any regulatory mechanisms would affect each allele identically. These conditions are approximated in intraspecific crosses, especially when genetically defined strains are used.

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Amphibian embryos are handy for experiments on hybridization since the gametes are easily obtained and the results can easily be observed. Several lines of evidence suggest that expression of nuclear genes in amphibians does not begin until just before gastrulation: nonviable hybrids between distantly related species proceed normally through blastula and arrest at gastrula (Brachet, 1960; Moore, 1955); in viable hybrids between more closely related forms, the developmental rate is typically maternal until gastrula, whereupon it becomes intermediate between the rates of the parental types (Moore, 1941); embryos with x-ray inactivated nuclei or treated with inhibitors of RNA synthesis will cleave but will not undergo gastrulation (Neyfakh, 1971); RNA synthesis, which is high during oogenesis, is shut down in the mature oocyte and during early cleavage (Brown, 1964) and resumes shortly before gastrulation (Brown and Littna, 1964; Brown and Gurdon, 1964).

The times of appearance of protein products resulting from nuclear gene expression during development should be related to the first appearance of the product of the paternal allele (since products of maternal alleles may be residually present from the egg) in embryos heterozygous for the protein studied. The analysis of reciprocal crosses between frogs from several populations in which different electrophoretic enzyme variants are found provide such data (Wright and Moyer, 1966, 1968; Wright and Subtelny, 1971; Johnson and Chapman, 1971a). An example of the analysis of an enzyme in a particular cross is shown in Figure 4. These data are summarized in Table 2. The time of expression of these enzymes is not at the gastrula stage but 68 hr or more later. In each case the first detection of paternal enzyme types corresponds to or precedes slightly a quantitative increase in enzyme activity in the embryo (Wright and Subtelny, 1973; Wallace, 1961). Similar results were obtained by using *Xenopus laevis* embryos heterozygous at the glutamate oxaloacetate transaminase (GOT) locus. The paternal expression occurs at Nieuwkoop and Faber's (1956) stage 37, gill circulation (Johnson and Chapman, 1971b). This stage is approximately the same stage as Shumway's stage 20 for *Rana*.

Procedures for culturing early mammalian embryos (Brinster, 1963; Mintz, 1964b) have made it possible to approach the problem of gene expression in mammalian development. RNA synthesis is detected in very early stages, two-cell to morula in mammalian embryos (Woodland and Graham, 1969; Piko, 1970; Mintz, 1964a; Epstein and Daentl, 1971). Incorporation of labeled precursors into protein increases between the eight-cell and blastocyst stages (Monesi and Salfi, 1967).

Enzyme activity measurements indicate that the early mammalian embryo is endowed with relatively high levels of LDH, G-6-PDH, and GPI (Epstein et al., 1969; Chapman et al., 1971). Activities of these enzymes decrease after 3 days of development before increasing at later stages. This

Fig. 4. Zymogram demonstrating isozymes produced by the LDH-B locus in hybrid frogs during various stages of development. The embryos are triploids containing two haploid sets of *Rana pipiens pipiens* chromosomes and one haploid set from *R.p. sphenocephala*. Products of LDH-B from pure *pipiens* and pure *sphenocephala* are seen in slots H and G, respectively. Hybrid embryo extracts are (a) stage 18-muscular response, (b) stage 19-heart beat, (c) stage 20-gill circulation, (d) stage 22-tail fin circulation, (e) stage 23-opercular fold, (f) stage 23 again (extract further diluted). Note the appearance of heterotetramers containing *sphenocephala* subunits at stage 19 and their increase with development (Subtelny and Wright, 1973).

suggests that enzyme degradation precedes new synthesis. A shift from LDH-1 to LDH-5 at implantation (Auerbach and Brinster, 1967) without an observation of LDHs 2, 3, or 4 indicates that LDH-1 (B-subunit) synthesis does not occur when LDH-5 (A subunit) is synthesized. Perhaps the LDH-B locus is expressed only in the growing oocyte. Appearance of LDH-5 indicates function of the LDH-A locus.

Increases in the activities before the blastocyst of fructose 1,6-

Table 2. Time of Expression of Various Enzyme Loci in *Rana* Hybrids

Enzyme Locus	Stage*	Characteristic	Time (Hr at 18° C)
6-PGD	18	Muscular response	92
LDH	18	Muscular response	110
IDH-S and MDH-m	19	Heart beat	120
GOT	19	Heart beat	130
MDH-S and GPI	20	Gill circulation	140
PGM	21	Cornea transparent	162
Aldolase—C	22	Tail fin circulation	192
ADH	24	Operculum closure	240

*Stages of development of *R. pipiens*, from the fertilized egg (stage 1) to feeding larva (stage 25) (Shumway, 1940).

Note: GOT is glutamate—oxaloacetate transaminase, and PGM is phosphoglucomutase.

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The time of expression of paternal alleles in heterozygous mouse embryos has been demonstrated for GPI by using gel electrophoresis (Chapman et al., 1971). Up until 4 days of development, the GPI phenotype is maternal. At day 4, enzyme activity is decreased, yet at day 5 (blastocyst), expression of a heterozygous GPI pattern is detected. Epstein et al., (1972) have demonstrated that 9-day mouse embryos, heterozygous at the IDH locus, have a heterozygous electrophoretic pattern. Most interestingly, this work disclosed that single embryos, or a portion of embryo from a backcross hybrid cross, could be analyzed. The authors point out the possibility of using enzyme markers that are closely linked to lethal genes to identify the mutants before they are morphologically distinguishable for biochemical, histological, or ultrastructural studies as well as for early detection and treatment.

Detection of electrophoretic variants of enzymes is not necessary for the analysis of the expression of polymorphic nuclear genes during embryogenesis. Neyfakh et al. (1973), for example, have used differences in the thermostability of aldolase from two different fish species (loach). The thermostability of aldolase from hybrids is intermediate between the parental type enzymes. In early hybrid stages the enzyme has the heat stability of the maternal species. At a stage of embryonic movement the heat stability of aldolase gradually changed in the direction of paternal species. Flickinger (1957) demonstrated that hybrid sea urchins developed an alkaline phosphatase activity intermediate to the parental types at about the gastrula stage. The maternal nature of sea urchin-hatching enzyme was demonstrated by using differences in Mn^{2+} sensitivity in the parental enzyme types (Barrett and Angelo, 1969).

Asynchronous Expression of Maternal Alleles

From the above we can see that the maternal form of the enzyme in heterozygous embryos precedes the presence of the paternal form and often dominates the electrophoretic pattern after paternal gene product is seen. This is not surprising since maternal enzyme produced during oogenesis may be expected to persist as maternal cytoplasm is partitioned into embryonic cells. However, how does one interpret the situation in which there is only expression of the maternal allele of an enzyme locus not previously seen in earlier embryonic stages, but later expression (or none) of the paternal allele?

This situation presents itself in the F_1 hybrids of crosses between ♀ rainbow trout (*Salmo irideus*) and ♂ brown trout (*S. trutta*). The products of the maternal allele (rainbow) for the loci LDH-A^1, LDH-A^2, LDH-C, and ADH were detected before the brown trout enzyme (Hitzeroth et al., 1968). The products of these loci were not seen in the eggs nor in very early

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embryonic stages, unlike the products of LDH-B^1, LDH-B^2, and PGM (Klose et al., 1969) which were, as was maternal 6-PGD in hybrid fish of the genus *Rutilus* (Klose and Wolf, 1970). The authors explain these results (amplified in a review by Ohno, 1969) as examples of asynchronous (earlier) activation of maternal, as opposed to paternal, alleles in heterozygotes. However, the time of expression of the rainbow and brown trout genes in the hybrids was consistent with the time in which they are expressed in the pure parental type embryos. Therefore, the earlier expression of rainbow alleles and later expression of brown alleles in the hybrids may not be due to their maternal versus paternal origin but perhaps because of their respective rainbow and brown troutness. To distinguish between these two possibilities, the critical experiment is the reciprocal cross (the minimum requirement in any claim for any sort of maternal effect). In such a cross the earlier expression of brown trout alleles would support the asynchronous activation hypothesis. However, the products of the ♀ brown X ♂ rainbow cross are not viable, which makes the claim from these data of earlier expression of maternal alleles in heterozygotes appear a bit premature. This is underscored because in other interspecific trout hybridization systems (in which reciprocal crosses are possible) the time of paternal gene expression is dependent on the direction in which the cross is made. After producing splake trout embryos by crossing ♀ lake trout (*Salvelinus namaycush* X ♂ brook trout (*S. fontinalis*), Goldberg et al. (1968) first detected the product of the paternal allele for LDH-A at 28 days after insemination. In the reciprocal cross, under the same conditions paternal allele product was not seen until day 32.

Interesting data relating to the asynchronous-activation hypothesis have been presented by Yamauchi (1972) and Yamauchi and Goldberg (1973) who used the splake system and took advantage of the polymorphism for G-6-PDH between the brook and lake trout. Here, F_2 embryos (crosses between two F_1 splake) were analyzed. All early embryos exhibited both G-6-PDHs, since both enzymes were produced in the F_1 oocytes. At hatching only either brook or lake G-6-PDH patterns were observed. The products of both alleles in heterozygotes (approximately half the F_2 fish as expected) were not equally expressed until much later—after complete yolk resorption. They suggest that the presence of either brook or lake G-6-PDH, but not both, at hatching indicates that residual oocyte product is no longer present. If true, half the F_2 should be homozygous for one or the other allele and would be expected to have only either brook or lake G-6-PDH. The other half of these embryos at hatching should be heterozygotes (as indicated by the expression of both types of G-6-PDH in half the F_2 after yolk reabsorption). Since those heterozygotes exhibit only a parental pattern, only one of the alleles (possibly maternal) was active with both producing protein later. Asynchronous activation of one or the other of the alleles may be indicated here. Another possibility (though remote) is that of differential degradation of one of the two residual oocyte products. Even if asynchronous activation is the answer,

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the conditions of the experiment do not allow one to test whether paternal or maternal alleles are preferentially or randomly activated.

An overriding complication in these interspecific crosses to determine the possibility of asynchronous allele activation is the very real possibility of nuclear-cytoplasmic incompatibility and its effect on various stages of gene expression. Indications of this possibility are seen in interspecific sunfish hybrids in which only the maternal allele for an esterase locus is expressed even in the adult F_1 (Whitt et al., 1972). A similar situation occurs in ADH activity in chicken-quail hybrids (Ohno et al., 1968). An analysis of such complications on maternal effects is possible on brief consideration of a few sea urchin hybrids. Various stages in the sequence of events from gene to phenotypic expression have been upset in different interordinal and interspecific hybrids: unequal replication of maternal versus paternal DNA, unequal transcription of maternal versus paternal RNA (Denis and Brachet, 1969a,b), and equal maternal and paternal replication of DNA and transcription of RNA with faulty translation of paternal message into protein (Whiteley and Whiteley, 1972; Ozaki and Whiteley, 1970; Badman and Brookbank, 1970). Therefore, the earlier or exclusive presence of maternal enzyme in hybrids may not be due to preferential maternal allele activation (transcription) but, depending on the case, may be due to abnormalities foisted on paternal gene products after the synchronous activation of maternal and paternal alleles.

The fact that it is possible to distinguish between residual oocyte gene products and the products of the embryonic genome in frog hybrids has been used to determine whether the earlier and dominating appearance of maternal enzyme is the result of preferential, asynchronous activation of maternal alleles. The analysis involves backcrossing or outcrossing female heterozygous for various enzyme loci (Wright and Moyer, 1968). Wright and Subtelny (1971) have applied this procedure to an analysis of androgenic haploid hybrids.

Androgenetic haploid hybrids are nuclear-cytoplasmic hybrids produced by removing the female chromosomes with a glass needle when they are visible shortly after fertilization (Porter, 1939). This procedure eliminates any contribution of the maternal allele to the enzyme pattern of the zygote. The nuclear paternal genes for enzymes are expressed at the same time in these cells as in the diploid hybrid controls. The pattern is strikingly different, however. The maternal type band of several enzymes is still dominant, despite the absence of maternal genetic material. No hybrid bands are present, which suggests that no concurrent synthesis of maternal and paternal subunits of the enzyme is taking place (Fig. 5). If a maternal mRNA stored in the cytoplasm were responsible for continued dominance of the maternal enzyme patterns, a hybrid band or bands would be seen in the haploid after paternal gene expression.

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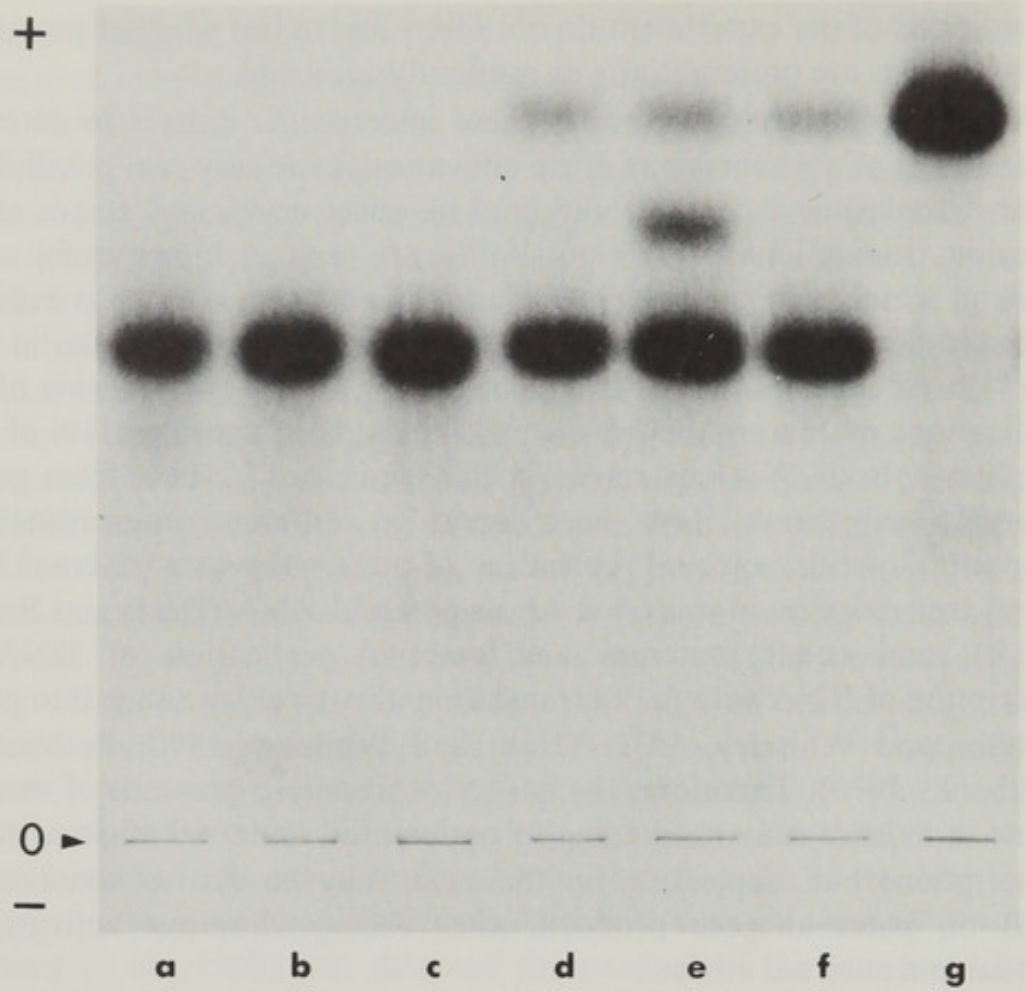
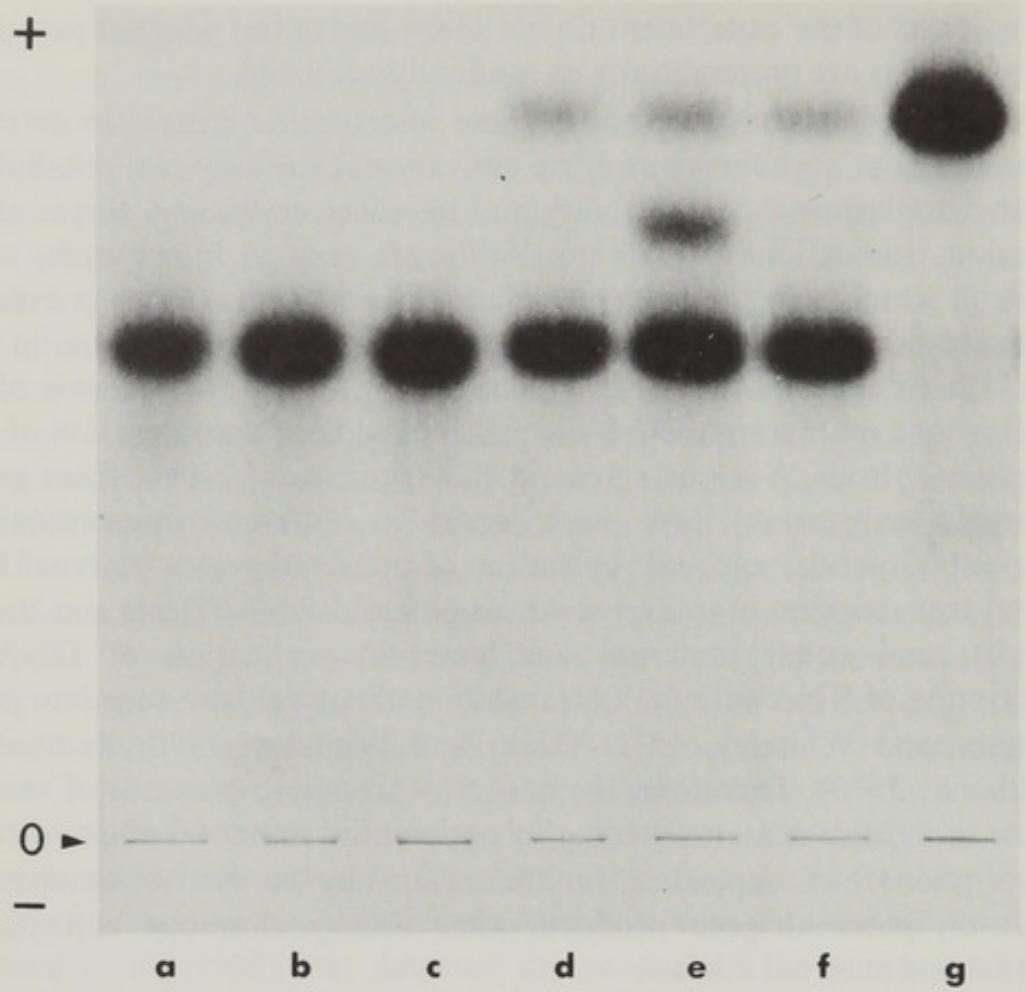


Fig. 5. Zymogram demonstrating 6-PGD isozymes produced by *Rana pipiens pipiens*, slot a; *R.p. berlandieri*, slot g; and their normal diploid hybrids, slots c and e; as well as their androgenetic haploid hybrids (female chromosomes removed), slots b, d, and f, at 34 (slots b and c), 118 (slots d and e), and 140 (slot f) hours of development. Note paternal 6-PGD dimer is expressed in the androgenetic haploid hybrids when the diploid control embryos show a hybrid 6-PGD pattern. However, no hybrid band is seen in the haploids (Wright and Subtelny, 1971).

An analysis of nuclear transplant hybrids confirms these findings. Polymorphic enzyme loci behave as if the time schedule of the host cytoplasm determines time of appearance of nuclear enzyme phenotypes. A nucleus of a *Rana pipiens* gastrula transplanted into an enucleated *R. p. sphenocephala* egg is nearly 48 hr older than the control nuclei of hybrids with the same eggs. Yet, the nuclear enzyme phenotype for LDH shows up not earlier, but on time with the host cytoplasmic system (S. Subtelny and D. A. Wright, 1973, to be published). These data clearly indicate that in heterozygotes produced between these closely related forms, the predominance of the maternal enzyme seen through early development is a function of residual oocyte enzyme and is not necessarily due to the preferential asynchronous activation of maternal alleles, nor to residual oocyte messenger RNA.



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GENE ACTION IN TUMORS HAVING A HEREDITARY BASIS

There are many advantages in using biochemical polymorphisms as markers for cell types in allophenic mice when the embryos used to produce the allophenics are from high and low tumor strains. One major advantage is that there is an opportunity to observe cells of high tumor disposition, and often in a low tumor environment. That is, as indicated in the summary of much of this work (Mintz, 1970), cells from hosts of high and low tumor susceptibility have been made to coexist throughout embryonic and adult life in different proportions in different tissues. Under these conditions, no matter what kind of tumor was observed, certain consistent patterns existed:

1. The tissue specificity, sex affected, age of onset, and number of tumor loci were the same in the allophenics as the high tumor controls.
2. The genotype of tissues surrounding tumor organs was not important in determining genetic susceptibility to tumors. In general, it was possible to conclude that intracellular rather than intercellular events were most important in the origin of the neoplasms.

It therefore becomes important to know what the intracellular basis for tumor susceptibility is. At least we might like to know how many genes are involved. In our laboratory we have been working with the platyfish-swordtail hybrid melanoma system as a model to answer this and other questions. Tumors in this system share certain characteristics with human tumors of suspected genetic etiology in general, such as early age of onset, multiple primary lesions, tissue specificity (Knudson et al., 1973). They also share certain characteristics with human hereditary melanoma in particular, such as histologic appearance (Erwin and Gordon, 1955; Grand et al., 1941), the effect of the sex of the individual (Siciliano et al., 1971; Allen, 1967), maternal effect in inheritance (Siciliano and Perlmutter, 1972; Anderson, 1972), and tyrosinase activity (Fitzpatric et al., 1950). This fish melanoma is strictly hereditary being produced only in certain interspecific crosses such as those between platyfish (*Xiphophorus maculatus*) carrying genes for normal macromelanophore spotting and swordtails (*X. helleri*). F_1 fish receiving the macromelanophore gene from the platyfish (all or half, depending on whether the platy is homozygous or heterozygous for the pattern) develop atypical melanosis or melanoma or both (see Fig. 6). Backcrossing the melanotic F_1 fish to swordtails produces a 1:1 ratio of fish with the macromelanophore gene to those without. Those without the gene develop no abnormality, but those with it become very severely melanotic (more than the F_1) at an early age. Backcrossing F_1 fish to platyfish produces fish less severely melanotic than the F_1. F_2 fish with the macromelanophore gene vary from least to most severely afflicted (see summaries by Atz, 1962 Anders, 1967).

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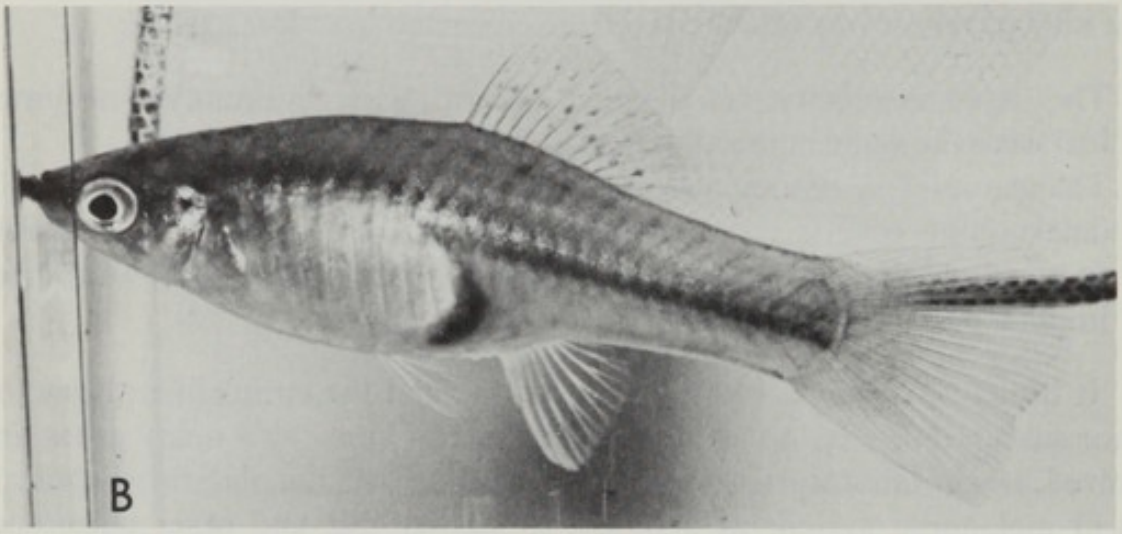
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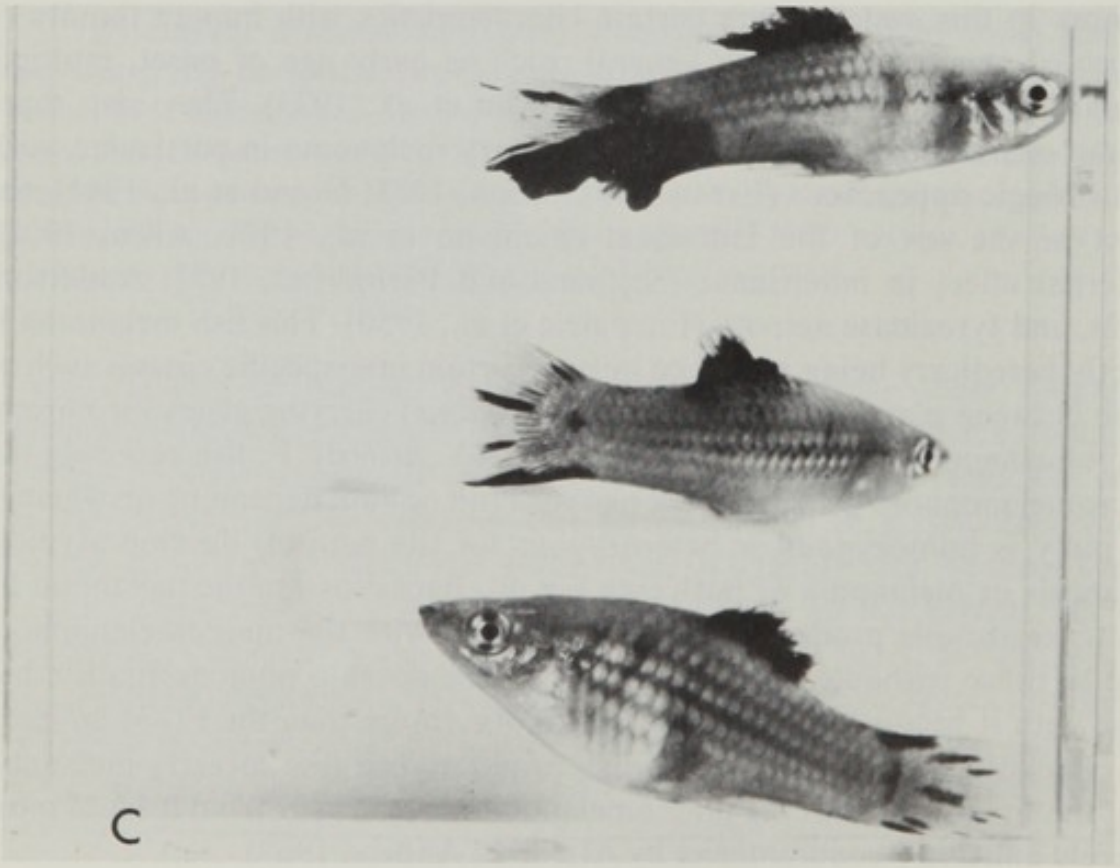
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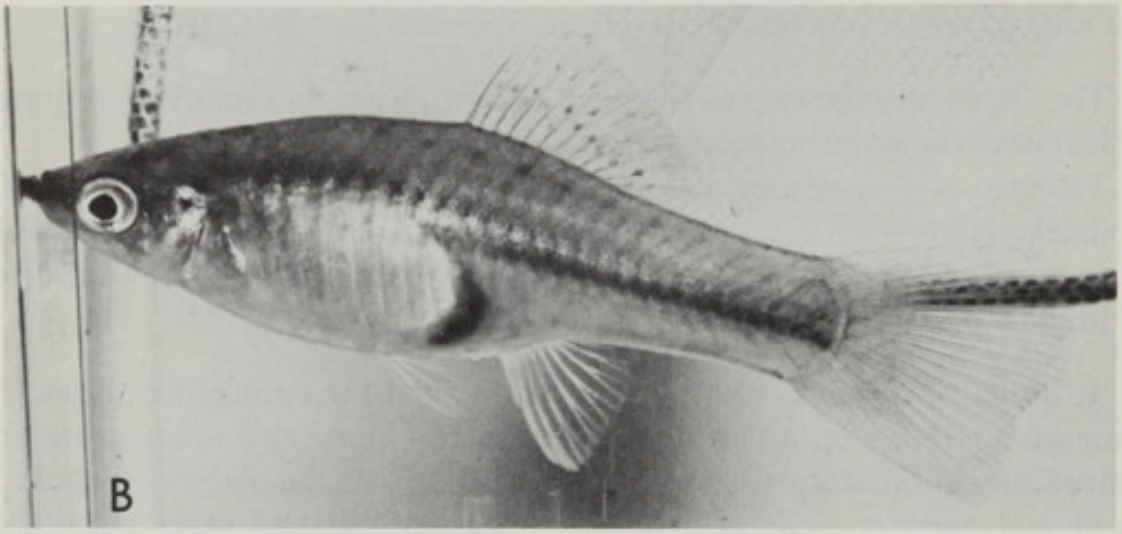
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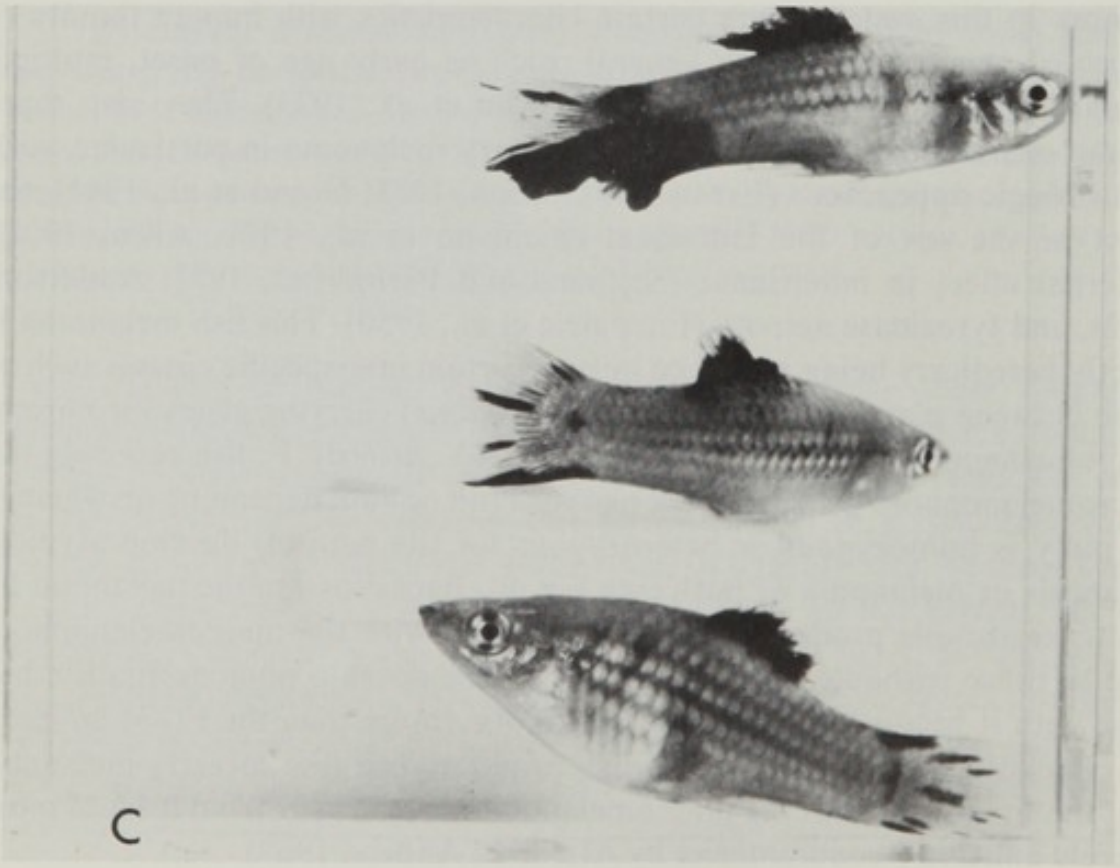
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This model therefore presents a relatively unique opportunity to study a tumor of purely genetic origin. However, the genetic basis of the neoplasm remains obscure—that is, whether it is due to factors from the swordtail that stimulate neoplasia, or to dilution of genes from the platyfish that control macromelanophore proliferation is not clear. There has been a lack of markers for identifying specific platyfish and swordtail chromosomes, so that they could not be indentified in the F_2 and backcross hybrids and related to the degree of abnormal melanosis. The use of biochemical polymorphisms now makes such a study possible.

The use of starch gel electrophoresis to analyze the isozyme genetics of the fish of this genus has enabled us to identify over 50 loci, 35 of which are polymorphic. Assortment of allozymes into backcross and F_2 hybrids indicate that swordtail and platyfish chromosomes can clearly be identified in them using these biochemical markers (Figs 1 and 7; Siciliano and Wright, 1973a; Wright et al., 1972; others in preparation). Once at least one marker is available for each chromosome ($n = 24$), the relationship of particular chromosomes to melanoma can be struck. Linkage analysis has been conducted on 13 of the polymorphic loci, and the available data indicate the identification of 10 linkage groups (Siciliano and Wright, 1973b).

EPILOGUE

"The proper study of mankind is man." Ultimately, yes. But there are limitations on human experimentation. The use of animal (and plant) models are as old as the science of biology. And, whatever the question being asked in the experiment, man the experimenter being ultimately self-centered is inevitably curious as to what the answer infers for his own welfare.

How useful are animal models? They may range from being totally meaningless to completely satisfying. For the most part, there are three categories of usefulness of animal models which may

1. Suggest mechanisms which operate in man;
2. Suggest experiments which may be carried out in man; and
3. Provide systems to experimentally approach problems in mechanisms which almost certainly also operate in man.

Fig. 6. Typical male platyfish (A), female swordtail (B) and their F_1 hybrids (C). Note the macromelanophore spotting pattern (*Sd*) on the dorsal fin of the platyfish and the absence of the trait in the swordtail. The F_1 fish are all of the same brood. The topmost F_1 is a male and shows more severe melanoma than the two females below it. Multiple sites of atypical melanosis can be seen in all F_1 hybrids receiving the *Sd* gene.

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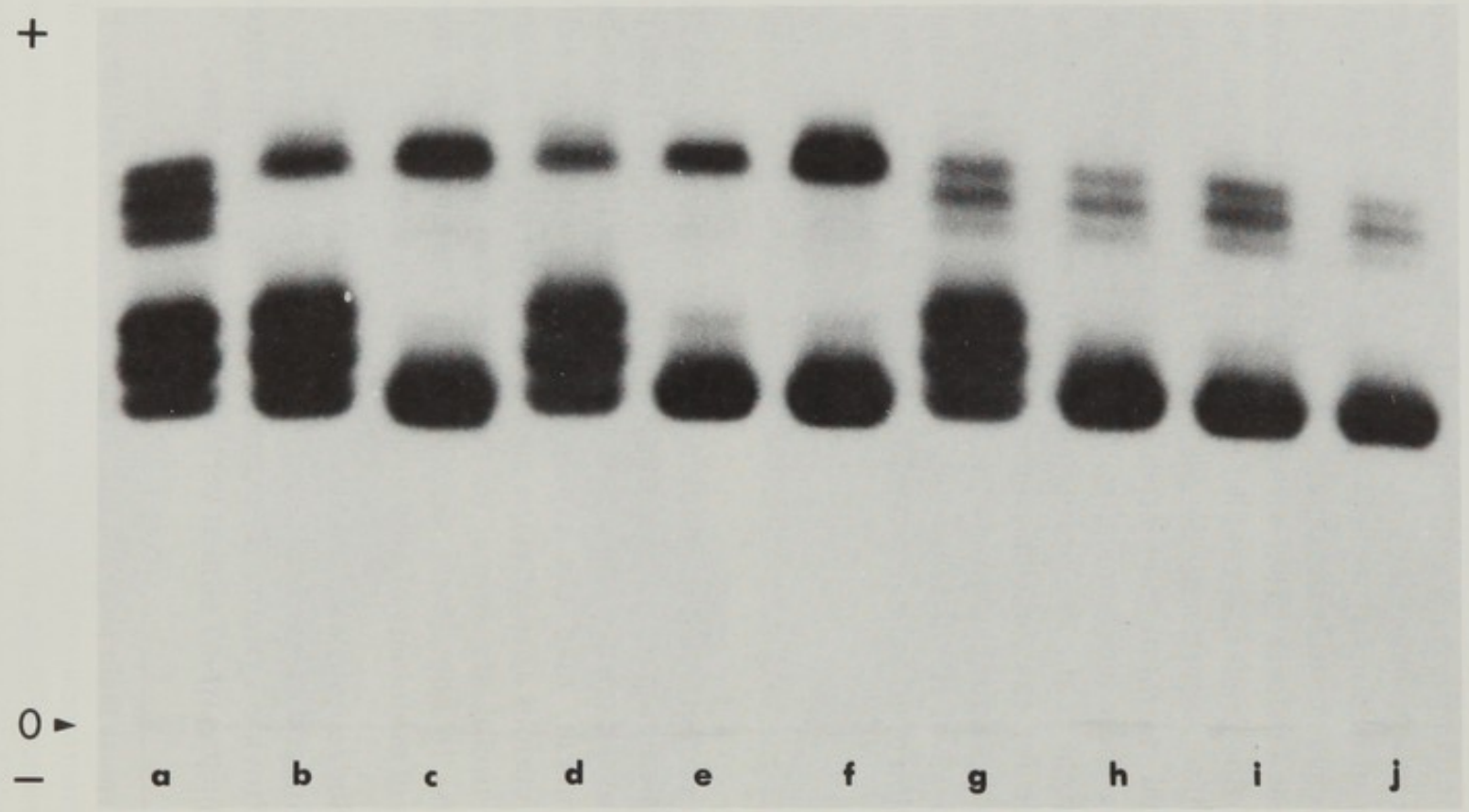
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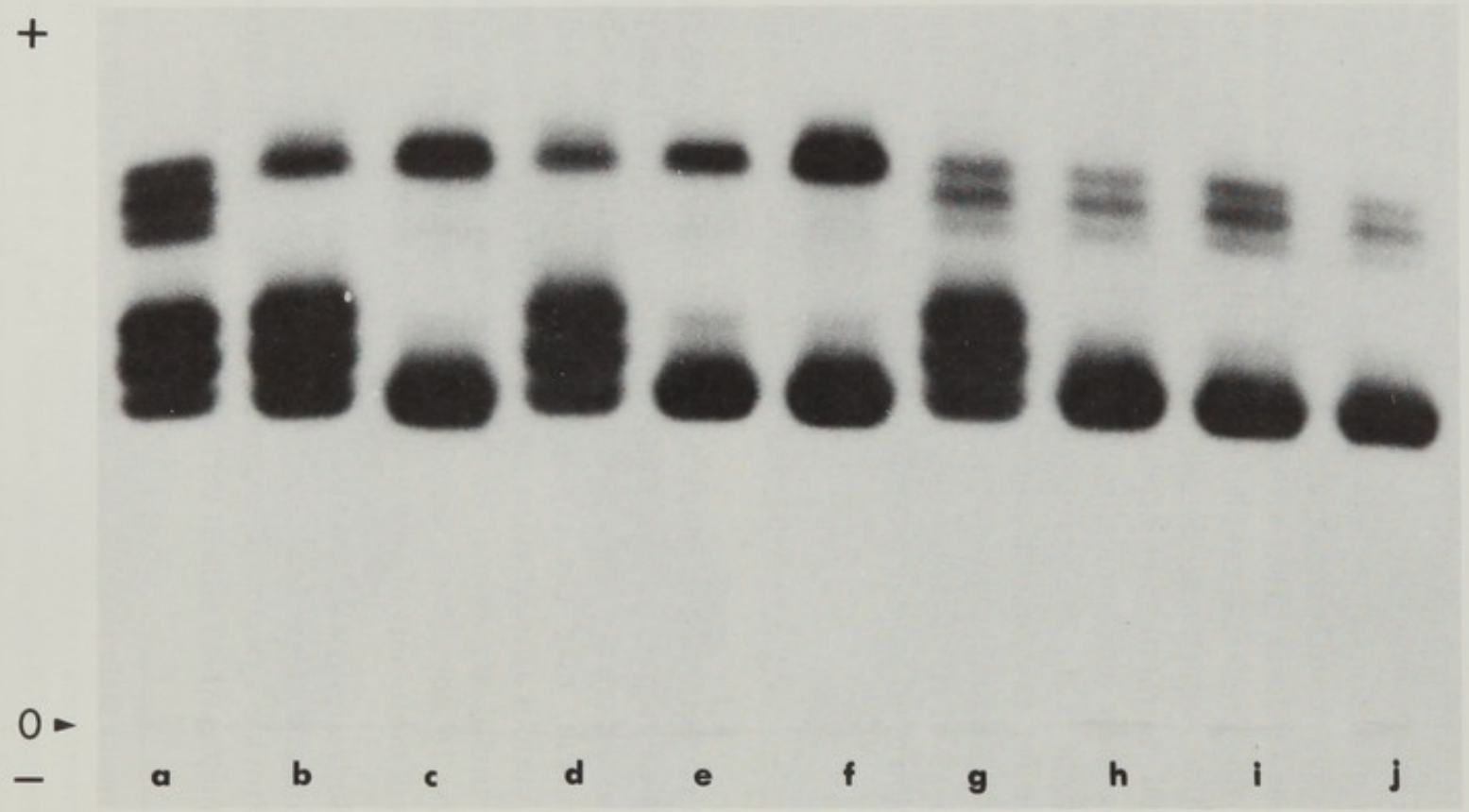
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The most useful models are, of course, those which directly provide answers about man or which lead to experiments that can be carried out in man, such as the hydroxylase genetics study. Least useful are those which provide suggestions but which cannot be directly verified. There are even dangers in such experiments—they may lead to extrapolations which are too attractive and which direct one's thinking along erroneous pathways, and can even block the way to correct lines of thought. An example is the model of gene control in bacteria—the Jacob-Monod theory. This beautiful and important theory has likely delayed progress in the study of gene control in eukaryotes.

There is another danger in the model-building game. In the present climate of government-supported research, with the added emphasis on relevance to man and target-oriented research, the scientist may find himself "reaching" to demonstrate the applicability of his project as a useful model for man. Sometimes this can become at least ludicrous if not downright dishonest.

Fig. 7. Zymogram demonstrating IDH isozymes from liver homogenates of 10 (a to j) backcross to swordtail hybrids. Two IDH loci function in liver of these fish. The products of locus 1 are faster (more anodal) than those of locus 2. For locus 1, the product of the swordtail allele is faster than the platyfish. For locus 2 the reverse is true. The female F_1 hybrid (produced by platyfish X swordtail) parent of these backcross fish was heterozygous at both loci: a pattern exemplified by a and g. The male swordtail parent of these fish was homozygous for swordtail type alleles at both loci, as seen in c, e, and f. Equal production of parental (hybrid only or swordtail only patterns at both loci: a, c, e, f, g) to recombinant (hybrid pattern at 1 locus with swotdtail pattern for the other: b, d, h, i, j) type individuals is consistent with these loci being most likely on different chromosomes (Siciliano and Wright 1973a). Whether any individual fish has both chromosomes for any locus from one or the other parental species or one chromosome from each can, therefore, be clearly determined.

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3

Inborn Errors of the Thyroid

John B. Stanbury

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INTRODUCTION 56
MAJOR WELL-DEFINED DEFECTS 56
Iodide Transport 56
Iodide Organification 59
Dehalogenase 64
Thyroglobulin and Iodothyronine Synthesis 66
OTHER INBORN ERRORS OF THE THYROID 72
Thyroglobulin Deficiency 72
TSH Unresponsiveness 72
Thyroxine-Binding Globulin and Goiter 72
Familial Cretinism with Muscular Hypertrophy 72
Thyroid Hormone Unresponsiveness 72
Familial Goiter with Intrathyroidal Calcification 73
SUMMARY 73
REFERENCES 74

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INTRODUCTION

The genetics of thyroid disease, including the inborn errors, has been reviewed extensively elsewhere (Fraser, 1969; Stanbury, 1972). Therefore, here we shall mainly consider the putative errors with respect to their genetic homogeneity, validity as separate entities, variety of expression, and with respect to our present level of understanding of the related abnormality. Only recent contributions to the literature are considered.

Only a small fraction of the inherited disorders of the thyroid are usually thought of as inborn errors. Graves' disease, lymphocytic thyroiditis, and nodular goiter show strong familial clustering, but the abnormal gene product, or even the point of impact of an abnormal gene product has hardly been suggested. By consensus, the inborn errors include only those disorders which are sufficiently well understood that one can identify the step in the metabolic process where the defect resides. In certain instances it is possible to identify an abnormal or missing gene product.

Often, by growing a goiter, the patient is able to compensate for deficient function and achieve a euthyroid state. This is possible in some, but in others the defect is so complete that adequate hormone synthesis is not possible and permanent hypothyroidism and retardation follow.

MAJOR WELL-DEFINED DEFECTS

Iodide Transport and the Transport Defect

Movement of iodide from plasma into the thyroid follicle is an active process (Wolff, 1972). The thyroid maintains a gradient which may vary from 20 to 1 or so, up to several hundred to 1, when the gland is stimulated. This gradient is lowered by a variety of metabolic poisons and by exposure to cold. In addition, transport displays saturation kinetics and may be competitively inhibited by a number of anions which have the same charge and similar partial molal ionic volumes as iodide. These findings all led to the inference that iodide transport is an active process; but the formal criteria for active transport require that there be a differential in electrical potential between the plasma and the components of the thyroid follicle. This was demonstrated by Woodbury and Woodbury (1963) when they found that the interior of the thyroid cell is approximately 40 to 50 mV negative to the outside of the cell and the follicular lumen. Thus, to move from the plasma to the interior of the thyroid cell, the iodide ion must move against an electrochemical gradient. From inside the cell it reaches the lumen of the follicle by moving with the electrical gradient.

The initial site of iodide accumulation is at the basal surface of the cell, as shown by Andros and Wollman (1967). Stimulation of the thyroid by thy-

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MAJOR WELL-DEFINED DEFECTS 56
Iodide Transport 56
Iodide Organification 59
Dehalogenase 64
Thyroglobulin and Iodothyronine Synthesis 66
OTHER INBORN ERRORS OF THE THYROID 72
Thyroglobulin Deficiency 72
TSH Unresponsiveness 72
Thyroxine-Binding Globulin and Goiter 72
Familial Cretinism with Muscular Hypertrophy 72
Thyroid Hormone Unresponsiveness 72
Familial Goiter with Intrathyroidal Calcification 73
SUMMARY 73
REFERENCES 74

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rotropic hormone or other agents lowers the electrical potential, and this hypothetically should favor movement of iodide into the thyroid follicle. However, manipulation of the potential difference by changes in external potassium concentration failed to affect iodide accumulation (Wolff, 1972), and it is not clear what, if any, the role of such factors might be in control of iodide transport (Wolff, 1972).

The existence of an iodide transport system in the thyroid suggests that there is a carrier for iodide in the plasma membrane. Evidence favoring the existence of such a carrier includes the saturation kinetics, the competition for transport by a large series of monovalent anions, and the increase in iodide transport following stimulation with thyrotropic hormone (TSH) (Isaacs and Rosenberg, 1967). Inhibition of TSH stimulation by inhibitors of protein synthesis suggests that transport is dependent on synthesis of a carrier (Knopp et al., 1970).

More direct evidence for a carrier has been obtained by identification of a phospholipid obtained from thyroid cells which binds iodide noncovalently (Vilkki, 1962), possibly in ionic binding in lipid micells in membranes (Posner and Ordonez, 1970). Iodide bound by the phospholipid is displaceable by anions which compete for iodide transport in the thyroid, and with similar potencies. Treatment of thyroid slices with lecithinase C impairs iodide transport (Macchia and Wolff, 1970). Furthermore, the distribution of this presumed carrier in various tissues—such as the salivary gland, choroid plexus, gastric mucosa, and mammary gland epithelium—corresponds to the capacity of those tissues to concentrate iodide (Wolff, 1972).

The various monovalent anions which compete for iodide have been tabulated and some of their properties listed by Wolff (1972). Some of these are selectively concentrated in the thyroid, whereas others such as thiocyanate are not, possibly because they are metabolized at so rapid a rate that selective concentration has not been demonstrated. The concentration of an anion in the medium which reduces the iodide concentration ratio by one-half can be used as a relative measure of the affinity of that ion species for the transport system. Perchlorate and pertechnitate are the most potent anions in this respect. They have an affinity for the transport system approximately two orders of magnitude greater than that for iodide. Iodide transport is dependent on an intact sodium pump, and is inhibited by inhibitors of Na^+-K^+-activated adenosine triphosphatase, such as ouabain.

When the iodide transport system is fully saturated, the ratio of iodide in the thyroid to that in the plasma, or to that in the suspending medium in experiments with slices or cell suspensions, approaches a value of approximately 0.4 (Wolff, 1972). This is the concentration ratio existing for other cells which have no selective concentrating capacity for iodide, but this low gradient does not preclude using iodide which enters the thyroid under these unfavorable conditions of transport for hormone synthesis.

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The complexity of the iodide transport system is indicated by the fact that cellular integrity appears to be necessary as well as energy flow. Thus, defective transport could derive from any of a number of possible disturbances, such as alterations in membrane structure, changes in structure of a presumed iodide carrier, or alterations in couplers relating cell energy flow to transport.

Defective transport of iodide has been assigned a causal role in hypothyroidism and goiter in a small number of patients (Stanbury and Chapman, 1960; Wolff et al., 1964; Gilboa et al., 1963; Papadopoulos et al., 1970). This interpretation has been based on the following findings in these patients: in spite of thyroid stimulation, only a small amount of iodide accumulates in the thyroid gland; the salivary secretion, which normally has an iodide concentration many fold that of the plasma, has an iodide concentration not significantly different from that of the blood; and, in some instances, administration of large doses of iodide has corrected the hypothyroidism and restored normal synthesis of thyroid hormone. All patients who have been reported with this syndrome have been hypothyroid when not receiving medication. Some have been permanently retarded, others not.

Usually the defect in transport of iodide in patients with this disorder has been quite complete. Papadopoulos et al. (1970) have reported their studies on a 9-year-old boy with goiter and retardation who had a partial defect of iodide transport. The patient was below the third percentile in weight, had a bone age of 5 years, and an intelligence quotient of 65. The ratio of iodide in the saliva to that in the plasma had a mean value of 17.2, which was far lower than the mean value of 84.3 in the authors' normal subjects. Serum thyroxine iodine concentration was 0.5 μg/100 cc. The concentration ratio of tissue slices prepared from the surgically excised thyroid was only 1.7, as compared to a normal value of 7.1 in their laboratory.

An interesting point at issue in this particular case was the retardation, because other patients with a complete transport defect have not been permanently retarded. The reason for this may have been that the patient had a very low intake of iodine, as indicated by a daily urinary excretion of only 16 μg. The thyroid clearance of iodide was 4.5 mm/min (normal, 8 to 40 mm/min), and the absolute iodide uptake by the thyroid was 0.7 μg/hr (normal, 0.5 to 6.0 μg/hr). Since the disease has been corrected in most patients by administration of large doses of iodine and since those patients resided in regions of iodine abundance, it seems reasonable to interpret the retardation in this patient with a partial transport defect as a result of a severer limitation on thyroid hormone synthesis imposed by a small amount of iodine available in the diet.

Similar considerations apply to the two retarded patients with familial goiter reported by Medeiros-Neto et al. (1972a). In both, the saliva/plasma

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ratio for ^{131}I was less than 8, and they came from a region in which iodine deficiency is recognized.

Diagnosis of defective iodide transport should rest primarily on a demonstration that iodide entry into the thyroid is limited or absent in spite of thyrotropic hormone stimulation. In addition, limited or absent iodide transport by the salivary glands should be demonstrated. Additional helpful information would be the finding that the iodine content of the thyroid is low. The information from one case and the speculations deriving therefrom might suggest that to make a diagnosis of a partial defect in thyroid iodide transport in a patient with hypothyroidism and goiter, one would require demonstration that there was limited iodine in the diet. Demonstration that administration of therapeutic doses of iodide restored hormone synthesis to normal would also be a strong point of evidence in favor of this diagnosis.

DeGroot (1970) has demonstrated lack of a transport system in "cold" thyroid nodules. He obtained four "cold" adenomas from which he was unable to establish a ratio of greater than 1 between radioiodide in tissue slices in vitro and in the suspending medium. On the other hand, these thyroids did form protein-bound iodine and synthesized labeled thyroglobulin. DeRubertis et al. (1972) have also studied "cold" thyroid nodules and found that they responded to thyrotropic hormone stimulation by an increase in adenyl cyclase activity, glucose-1-^{14}C oxidation, and phosphate incorporation into phospholipids, but not in iodide transport. Indeed, these adenomas responded better than did adjacent normal thyroid tissue. Thus, these nodules had an intact response to thyrotropin (TSH) in several aspects of thyroid function, but not in iodide transport, possibly because of failure of cyclic adenosine monophosphate (AMP) to affect the transport aspect of thyroid iodine metabolism.

Disorders of Iodide Organification

Iodide within the thyroid epithelial cell must lose an electron in order to displace a proton from the aromatic ring of an iodotyrosyl residue in thyroglobulin (i.e., organification). Because of the high redox potential of the iodide/iodine system, probably the only oxidizing agent in the cell which could oxidize iodide is peroxide. Peroxidase activity in the thyroid has been recognized for a long time. During recent years a number of investigators have obtained tissue preparations from the thyroid with peroxidase activity, and sufficiently pure preparations have been obtained for detailed study (Taurog, 1970; Ljunggren and Akeson, 1968; DeGroot et al., 1965; Hosoya and Morrison, 1967).

The molecular weight of bovine thyroid peroxidase has been assigned a value of 62,000 by Taurog, as compared to an assigned molecular weight of approximately 42,000 for horseradish peroxidase. The thyroid peroxidase is

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a membrane-bound hemoprotein. The heme spectrum of thyroid peroxidase is not identical to that of horseradish peroxidase. Furthermore, and in contrast to horseradish peroxidase, the heme of thyroid peroxidase is not covalently bound and can be extracted. Taurog (1970) has estimated the turnover number of bovine peroxidase to be greater than 13,000. He has studied iodination of protein using several peroxidases including thyroid peroxidase. There are differences in pH optimum and in yield of iodoamino acids. For activity the bovine peroxidase requires peroxide and an acceptor. Tyrosine, bovine serum albumin, and thyroglobulin are acceptors, as is monoiodotyrosine. In model experiments investigators observed an optimal concentration of iodide and obtained evidence that iodination of substrate protein proceeds too rapidly for free iodine to be the iodinating species. Thus, it appears that the role of peroxidase is not simply that of providing free I_2 which would then iodinate protein noncatalytically. Probably I_2 or an equivalent species is formed on the surface of the peroxidase. Evidence suggests that a sulfenyl iodide is formed which is the iodinating component. Unless there is a transfer agent for the activated iodine from the peroxidase to its reaction site, thyroglobulin iodination would require a binary collision, presumably at the apical border of the thyroid epithelial cell, where other evidence suggests that the peroxidase is primarily located (Strum et al., 1971). In any case, since several peroxidases, including that from the bovine thyroid, rapidly iodinate thyroglobulin in the presence either of peroxide or of a peroxide generating system, it is unnecessary to postulate a carrier or transfer enzyme, such as a "tyrosine iodinase." The peroxidase itself seems to fulfill this function.

A large number of flavoprotein enzymes could provide the hydrogen peroxide needed in the oxidation of iodide. These react directly with O_2, reducing the oxygen to peroxide. Among these are L-aminoacid oxidase, aldehyde oxidase, and xanthine oxidase. Which of these or others might be most important in the supply of peroxide in the thyroid cell has not been determined. Ljunggren and Akeson (1968) solubilized a fraction from bovine thyroids using deoxycholate and trypsin. Their preparation had a molecular weight of approximately 45,000 and a turnover number of 150,000/min. It was a heme protein. DeGroot et al. (1965) studied a similar preparation and found it was protected by noncovalently linked iodide.

The role of peroxidase in iodination reactions in the thyroid is highlighted by the effects of various antithyroid drugs on peroxidase-catalyzed iodination. Drugs such as propylthiouracil inhibit the enzyme activity, but precisely how this inhibition occurs has not been established. Various explanations have been proposed, such as formation of a peroxidase-iodinium complex for which the antithyroid drug and tyrosyl groups compete (Morris and Hager, 1966), or formation of a sulfenyl iodide complex which reacts with the antithyroid drug with reduction of the iodine (Jirousek and Cun-

a membrane-bound hemoprotein. The heme spectrum of thyroid peroxidase is not identical to that of horseradish peroxidase. Furthermore, and in contrast to horseradish peroxidase, the heme of thyroid peroxidase is not covalently bound and can be extracted. Taurog (1970) has estimated the turnover number of bovine peroxidase to be greater than 13,000. He has studied iodination of protein using several peroxidases including thyroid peroxidase. There are differences in pH optimum and in yield of iodoamino acids. For activity the bovine peroxidase requires peroxide and an acceptor. Tyrosine, bovine serum albumin, and thyroglobulin are acceptors, as is monoiodotyrosine. In model experiments investigators observed an optimal concentration of iodide and obtained evidence that iodination of substrate protein proceeds too rapidly for free iodine to be the iodinating species. Thus, it appears that the role of peroxidase is not simply that of providing free I_2 which would then iodinate protein noncatalytically. Probably I_2 or an equivalent species is formed on the surface of the peroxidase. Evidence suggests that a sulfenyl iodide is formed which is the iodinating component. Unless there is a transfer agent for the activated iodine from the peroxidase to its reaction site, thyroglobulin iodination would require a binary collision, presumably at the apical border of the thyroid epithelial cell, where other evidence suggests that the peroxidase is primarily located (Strum et al., 1971). In any case, since several peroxidases, including that from the bovine thyroid, rapidly iodinate thyroglobulin in the presence either of peroxide or of a peroxide generating system, it is unnecessary to postulate a carrier or transfer enzyme, such as a "tyrosine iodinase." The peroxidase itself seems to fulfill this function.

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ningham, 1968). Peroxidase might catalyze formation of a free tyrosyl radical (Yip and Hadley, 1967) which then might react with the antithyroid drug. It only seems entirely established that free iodine is not the iodinating species. Taurog (1970) has suggested that the inhibitory action of the antithyroid drugs can best be explained as an interference with the formation of the active iodinating species, rather than as competition for oxidized iodine. The antithyroid drugs of the thiourea type are, in fact, accumulated in the thyroid (Marchant et al., 1972).

The available information, therefore, suggests that iodide is oxidized to an iodinating complex by a peroxidase present in highest concentration at the apical border of the thyroid epithelial cell. Iodination of aromatic residues in thyroglobulin proceeds without free I_2 as intermediary, but rather appears to be catalyzed by the peroxidase (Lamas et al., 1972). If so, at least two reactions appear to be proceeding on the peroxidase molecule: the peroxidation of iodide and the transfer of the iodide to a receptor site on thyroglobulin. One might surmise that the requirements for optimal activity are integrity of the peroxidase, free flow of peroxide and iodide, and structural integrity of the substrate, thyroglobulin, and in addition absence of any interferring substance such as a thiourea-like drug, excessive catalase, or any kind of compartmentalization which might interfere with ready access of the components of the system.

The activity of peroxidase in the normal thyroid is not rate-limiting in organification of iodide. Almost all the iodide which enters the gland becomes protein-bound at once, and administration of thiocyanate or perchlorate shortly after radioiodide results in no detectable loss of iodide from the gland. When the process of organification is blocked, as with thiouracil, then administration of these causes a precipitous loss of accumulated iodide from the gland.

Rapid discharge of labeled iodide from the thyroid after administration of perchlorate or thiocyanate has been interpreted as an indication of impaired organification. This test has been used as a primary indicator of a defect in iodide organification in at least three clinical settings. Certain rare patients with familial goiter and hypothyroidism have been described who discharge most of an accumulated dose of radioactive iodine within a few minutes after administration of a few hundred milligrams of perchlorate or a gram or two of thiocyanate. The same response has been described in patients without severe hypothyrodism. Partial discharge of iodide has also been observed in patients with Pendred's syndrome and this has been attributed to a partial inhibition of iodide organification characteristic of that disorder. Finally, most patients with lymphocytic thyroiditis can demonstrate a modest discharge of iodide.

The patients who were originally assigned to the category of "organification defect" were severely cretinous and appeared to have a com-

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plete defect in organic binding of iodide (Stanbury, 1972). In addition, the concentration of iodide in the thyroid was extremely low, and kinetic analysis of iodine metabolism was entirely consistent with an absence of bound iodine in the gland. More recently, patients have been described who are much less seriously retarded and in whom the defect appeared to be only partial. Most recently, the heterogeneity within this category of patients has been disclosed.

Precipitous discharge of labeled iodine from the thyroid after administration of thiocyanate or perchlorate has become generally accepted as the central criterion for impaired iodide organification in the thyroid. No guide lines have been established for exactly how precipitous the discharge must be or what fraction of the labeled iodide in the thyroid must disappear from the gland before one may assume defective organification (Marie et al., 1969; Jaffiol et al., 1969a). Pentschew et al. (1971) found that 13.7 percent of 675 patients with endemic and sporadic goiter discharged at least 10 percent of accumulated labeled iodine after 1 gm ClO_4^-. Since, in the normal person, there is virtually no detectable drop in labeled iodine in the thyroid after administration of perchlorate or thiocyanate, a drop beyond the statistical error of counting suggests impaired organification. At least two problems may arise. Impaired absorption after oral administration of thiocyanate or perchlorate may obscure the results. More important, rapid turnover of radioiodine by the thyroid may cause a recorded drop in measured labeled iodine in the thyroid after administration of the blocking anion. This latter difficulty may be resolved by serial measurement of labeled protein-bound iodine. If the fall in counts over the thyroid is due to this problem, one should observe a high level of protein-bound labeled iodine. Still another unresolved problem is the time at which the thiocyanate or perchlorate should be given after the labeled radioiodide. If the defect in organification is partial, then the longer one waits after administering the iodine, the less likely there will be a significant, demonstrable pool of dischargeable iodide in the thyroid. On the other hand, the earlier the test is performed, the more likely there will be confusing background counts from blood circulating through the thyroid, particularly in patients with low uptake.

Three types of patients with congenital goiter who appear to have impaired organification of iodine have been described. The first have severe hypothyroidism, goiter, no hearing defect, and virtually complete discharge of iodine after administration of thiocyanate or perchlorate (Stanbury, 1972). A second group, originally described by Clayton et al. (1958) are similar except that the discharge following anion is only partial, and the patients are euthyroid or only mildly hypothyroid. Similar patients have more recently been described by Furth et al. (1967). The third type belongs to the Pendred syndrome group. These patients are either euthyroid or

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mildly hypothyroid, are deaf, and have a partial defect in iodide organification, as indicated by the perchlorate discharge test.

Haddad and Sidbury (1959) were the first to demonstrate an absence of peroxidase activity in the thyroid of a patient with familial goiter, hypothyroidism, and virtually complete thiocyanate discharge of thyroid iodide. Recently Hagen et al. (1971) and Niepomniszcze et al. (1972) studied a 16-year-old patient who was euthyroid and who had normal hearing, but had familial goiter and approximately a 50 percent release of thyroid-labeled iodine after administration of perchlorate. Extensive studies on the thyroid gland after surgical excision disclosed an absence of peroxidase activity as measured by several methods, including the ability of a solubilized preparation to iodinate tyrosine, to form triiodide, to peroxidize guaiacol, and to convert trihydroxyphenol pyruvic acid into an unidentified product in the presence of peroxide. When the inactive enzymatic preparation was incubated with hematin, activity was restored to normal levels in a tyrosine iodinase assay, but not in the triiodide assay. A possible explanation offered for the failure of hematin incubation to restore peroxidatic activity in the triiodide assay was that the peroxidatic activity toward iodide in that assay was more labile to peroxide. The conclusion was that in this type of organification defect, the fault lay in disordered structure of the thyroid peroxidase which made the noncovalent binding of hematin less secure and also rendered the molecule more labile to peroxide. The suggestion was offered that in patients with complete organification defect, the peroxidase is either absent or entirely nonfunctional. More recently, Niepomniszcze et al. (1973) studied a 14-year-old goitrous hypothyroid girl with complete perchlorate discharge of iodide. There was no restoration of peroxidase activity when thyroid tissue was incubated with hematin. Restoration of enzymatic activity by addition of a prosthetic group is analogous to that in cystathionuria; in some patients with that disease, addition of an unusually large amount of the coenzyme pyridoxine largely restores the activity of cystathionase in vivo (Frimpter, 1972). Valenta et al. (1972) have also reported in abstract a patient with cretinism and goiter and without demonstrable peroxidase activity, as measured using guiaicol, iodide, and thiocyanate as electron donors. Interestingly, peroxidase activity was demonstrable in the saliva. Unfortunately, restoration of activity by hematin was not tested.

The soluble proteins of a goiter from a retarded patient with organification defect have been examined by Mouriz et al. (1969). A protein precipitable with antithyroglobulin antiserum was found which contained no iodine. Medeiros-Neto et al. (1972b) have reported a retarded child with goiter and organification defect who also had muscular hypertrophy. Other patients with organification defect are described by Marie et al. (1969).

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Patients with lingual thyroids and hypothyroidism and positive perchlorate tests have been reported by Smith et al. (1966) and by Ferrini and Biassoni (1966). It is often difficult to distinguish between a positive perchlorate test arising from discharge of labeled iodide from the gland and rapid turnover of iodine in a hyperfunctioning remnant or rest. The potential malignancy of nodules developing in the thyroid of patients with an organification defect has been noted by Salabe et al. (1969). Valenta (1966) studied a patient with carcinoma of the thyroid. Accumulated iodine was completely discharged after administration of perchlorate.

The fundamental defect in the Pendred syndrome has not been identified. Although partial discharge of labeled iodine from the thyroid of these patients by perchlorate or thiocyanate suggests a partial organification defect, in at least two patients belonging to this group, high levels of peroxidase activity were found (Ljunggren and Vecchio, 1969; Ljunggren et al., 1973). A chance observation of a persistent blocking effect of perchlorate on iodide transport into the thyroid in one patient suggests that these patients may have an unduly prolonged effect from the anion (Stanbury, unpublished).

The origin of deafness in Pendred's syndrome is unclear. Changes have been induced in the middle ear of developing chicks by injection of propylthiouricil into the yolk sac (Bargman and Gardner, 1972). This observation suggests that thyroid function is necessary for the development of the middle ear in the chick, or that oxidation of iodide is necessary for some other process unrelated to thyroid function in ear development, or that the drug has an effect on the ear independent of its effect on iodine metabolism.

Several recent publications have dealt with the inheritance pattern of the Pendred syndrome and some of its clinical features (Visalakshmi and Khanna, 1969; Fraser, 1969; Thompson et al. 1970). Although in most instances the pattern is that of an autosomal recessive with complete concordance between goiter and deafness, there are reports of dissociation of deafness and goiter within kindreds (Papasov, 1969). In some instances there are patients who are only partially deaf, and in some the deafness is much more severe on one side than on the other. Thyroglobulin, iodoalbumin, and an insoluble iodoprotein have been identified in the thyroids of patients with this syndrome (Mederios-Neto et al., 1968; Milutinovic et al., 1969).

The Dehalogenase Defect

When thyroglobulin is degraded to its constituent aminoacids in the thyroid, thyroxine and triiodothyronine are released into the blood, but mono- and diiodotyrosine are largely deiodinated and the iodide reused once again in the iodination of thyroglobulin. If iodotyrosine deiodination is in-

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hibited by dinitrotyrosine, iodotyrosines are secreted by the thyroid (Greer and Grimm, 1968). The dehalogenating enzyme is nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and its action is inhibited if NADPH is oxidized (Bastomsky and Rosenberg, 1966). Some of its properties have been studied (Matsuzaki and Suzuki, 1967). Deiodination of iodotyrosine by thyroid tissue preparations is enhanced by flavin adenine dinucleotide (FAD), and the latter may be involved as a cofactor in the reductive deiodination of iodotyrosines in vivo (Rosenberg and Ahn, 1969). Deiodination of mono- and diiodotyrosine can also be demonstrated in preparations from other tissues such as liver and kidney. The dehalogenase is also found in small amounts in the soluble fraction from tissue cells and may be different from the particle-bound enzyme (Kusakabe and Miyake, 1966).

The patients who were originally characterized as lacking deiodinase activity had this deficit not only in the thyroid but in other tissues, as well, and the deficiency seemed to be complete. No evidence was found of any deiodination of injected mono- or diiodotyrosine (Stanbury, 1972). Impairment in deiodination of intravenously administered labeled diiodotyrosine by some family members was interpreted as evidence that they were heterozygous for the same defect, and others have made similar observations (Niall et al., 1968; Murray et al., 1965). Jaffiol et al. (1969b) have suggested a rapid, standardized, intravenous diiodotyrosine (DIT) test.

More recently, families of patients have been described with partial deficiencies of dehalogenase activity (Larkander et al., 1971). Patients have also been described who had no deiodinase activity in the thyroid, but who had it in other tissues (Kusakabe and Miyake, 1963), as well as some who had no deiodination in other tissues but had deiodinating activity in the thyroid (Kusakabe and Miyake, 1964). Lissitzky et al. (1968) have described a patient without iodinating activity and with a large amount of iodinated albumin in the thyroid gland, whereas Thomson and McGirr (1969) have reported patients similarly studied who had no iodoalbumin detected either in the thyroid or in the peripheral blood.

The initial conceptual formulation of this disorder of the thyroid led to the hypothesis that treating the patient with iodide should restore the ability of the thyroid to produce a normal amount of thyroid hormone and would reduce the goiter. The demonstration of the validity of this hypothesis by Choufoer et al. (1960) has been amply confirmed in more recent studies (Niall et al., 1968). The iodide concentration of the blood appears to govern the rate of hormone synthesis in these patients. Harden et al. (1967) found that at a concentration of 1.0 μg/100 cc, hormone synthesis proceeded normally when the intake of iodide was increased to 612 μg/day. Lissitzky et al. (1965) found that the administration of 1.25 mg iodine daily restored thyroid function to normal. Since many persons in the United States receive this

amount of iodide daily because of the widespread use of iodine in the bread-making and other industries, one would not expect this disorder to become clinically evident often.

From the foregoing, we can see that what appeared at the time of the initial studies to be a simple monotropic disorder, now is resolving into a genetically heterogeneous group of disorders perhaps expressing multiple alleles, but manifest through common clinical and biochemical manifestations.

Disorders of Thyroglobulin and Iodothyronine Synthesis

From time to time a number of patients with familial goiter have been characterized variously as having impaired coupling of iodotyrosyl residues to form thyroxine and triiodothyronine, or impaired synthesis of thyroglobulin, or as patients identifiable uniquely by the presence of an abnormal iodinated peptide in the peripheral blood. The evidence that these are three separate and distinct entities is not as strong as previously implied.

An iodinated peptide has been detected in the peripheral blood in several different disorders of the thyroid. For example, an iodinated albumin-like protein (nonbutanol-extractable) constitutes a large fraction of the precipitable iodine of the blood of patients with Hashimoto's disease or Graves' disease, and it has been detected in certain patients with simple goiter. Some investigators suggest that when the thyroid becomes hyperplastic, albumin from the plasma circulates through the thyroid and penetrates the thyroid cells where it serves as a substrate of iodination when the normal substrate is in short supply. Thus, the presence of an iodinated albumin-like component in the blood does not in itself indicate or specify an inborn error of the thyroid, but may only be indicative of hyperplasia. On the other hand, all patients with hyperplastic thyroids do not have an iodinated peptide in the peripheral blood (Thomson and McGirr, 1969).

A large fraction of the iodoprotein in the thyroid glands of patients with familial goiter and nonbutanol-extractable iodine in the plasma is usually an albumin-like component or, in any event, an iodoprotein different from thyroglobulin. Thus, it is possible that the syndrome of familial goiter with nonbutanol-extractable plasma iodine may arise from unpaired thyroglobulin synthesis, which in turn results in hyperplasia and formation of nonthyroglobulin iodoprotein, especially iodoalbumin.

One might expect that errors in thyroglobulin synthesis and structure exist and that these might be detected by physical and chemical means, just as errors in hemoglobin structure have been detected. An error in primary structure might prevent the evolution of normal tertiary folding of the molecule, with the result that subunits of finished thyroglobulin would appear in the gland, or alteration of structure might change the conformation

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Disorders of Thyroglobulin and Iodothyronine Synthesis

From time to time a number of patients with familial goiter have been characterized variously as having impaired coupling of iodotyrosyl residues to form thyroxine and triiodothyronine, or impaired synthesis of thyroglobulin, or as patients identifiable uniquely by the presence of an abnormal iodinated peptide in the peripheral blood. The evidence that these are three separate and distinct entities is not as strong as previously implied.

An iodinated peptide has been detected in the peripheral blood in several different disorders of the thyroid. For example, an iodinated albumin-like protein (nonbutanol-extractable) constitutes a large fraction of the precipitable iodine of the blood of patients with Hashimoto's disease or Graves' disease, and it has been detected in certain patients with simple goiter. Some investigators suggest that when the thyroid becomes hyperplastic, albumin from the plasma circulates through the thyroid and penetrates the thyroid cells where it serves as a substrate of iodination when the normal substrate is in short supply. Thus, the presence of an iodinated albumin-like component in the blood does not in itself indicate or specify an inborn error of the thyroid, but may only be indicative of hyperplasia. On the other hand, all patients with hyperplastic thyroids do not have an iodinated peptide in the peripheral blood (Thomson and McGirr, 1969).

A large fraction of the iodoprotein in the thyroid glands of patients with familial goiter and nonbutanol-extractable iodine in the plasma is usually an albumin-like component or, in any event, an iodoprotein different from thyroglobulin. Thus, it is possible that the syndrome of familial goiter with nonbutanol-extractable plasma iodine may arise from unpaired thyroglobulin synthesis, which in turn results in hyperplasia and formation of nonthyroglobulin iodoprotein, especially iodoalbumin.

One might expect that errors in thyroglobulin synthesis and structure exist and that these might be detected by physical and chemical means, just as errors in hemoglobin structure have been detected. An error in primary structure might prevent the evolution of normal tertiary folding of the molecule, with the result that subunits of finished thyroglobulin would appear in the gland, or alteration of structure might change the conformation

of the molecule in such a way that its physical properties would be altered or that iodination or coupling would be impeded.

The structure of the thyroglobulin molecule is such that under normal circumstances only a small proportion of the tyrosyl residues become coupled to form iodothyronines. Although information regarding the three-dimensional requirements for optimal coupling is limited, Dunn (1970) has suggested that only those tyrosyl residues next to serine are easily formed into thyroxine. Thus, a change in amino acid sequence in thyroglobulin might result in impeded coupling and iodothyronine synthesis, or it might result in an altered rate of thyroglobulin synthesis, or in failure of the molecule to be formed at all. Hypothetically, impaired thyroglobulin synthesis could result in a so-called coupling defect, or in the syndrome of plasma nonbutanol-extractable iodine, or it could be recognized as a primary disorder in thyroglobulin synthesis.

A number of patient studies have convincingly established the existence of thyroid disease arising from impaired or absent thyroglobulin synthesis. Alexander and Burrow (1970) studied three cretins with goiter. The thyroids of these patients could effectively transport and oxidize iodide. Most of the iodine was bound to an albumin-like protein in the thyroid. There was evidence for only a small amount of thyroglobulin. Although the total iodine concentration of these cretinous glands was somewhat less than half normal, the total iodine content was presumably larger than normal because of the size of the gland. After labeling, only 2 percent of the radioiodine in the thyroid was present as thyroxine. Protease activity, when tested against normal thyroglobulin, was normal. Only a small amount of thyroglobulin was detected either by salt fractionation, DEAE cellulose chromatography, radioimmunoelectrophoresis, or sucrose gradient centrifugation. Similar studies have been reported by Cassano (1971) and from this laboratory (Riddick et al., 1969).

Quite recently Lissitzky et al. (1973) have reported two congenitally hypothyroid subjects who had mostly albumin in the thyroid. An iodinated component that reacted with antithyroglobulin antibody was present, but it had a sedimentation constant so low as to suggest that it might be a small fragment of thyroglobulin which failed to undergo normal maturation. These patients had no noteworthy iodoprotein in the peripheral blood. In an important recent study, Savoie et al. (1973) reported four patients with congenital goiter and hypothyroidism who had no thyroglobulin in their thyroids, abundant albumin, and a large daily excretion of mono- and diiodohistidine. These iodoaminoacids may have been missed in previous studies. They were also found in peptide-linked form in the thyroid, and may be customarily formed and excreted in patients in whom thyroglobulin, the usual substrate of iodination, is reduced or absent.

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A number of other studies have indicated either absence of thyro-

globulin or a diminished amount coupled with a predominant iodinated protein with properties quite different from thyroglobulin. Among these have been iodoproteins unusually resistant to tryptic digestion, iodoproteins more soluble and less soluble in ammonium sulfate than thyroglobulin (Michel et al., 1964), and iodinated proteins with altered sedimentation or immunological properties (cf, Stanbury, 1972). Both Falconer et al. (1970) and Rac et al. (1968) have studied Merino sheep which appear to have impaired synthesis of thyroglobulin, but Falconer et al. (1970) detected a minor component that is an immunoprecipitable thyroglobulin-like iodoprotein in hyperplastic glands. In some instances the concentration of protein-bound iodine (PBI) in the plasma has been elevated above normal (Furth et al., 1970; Rac et al., 1968; Katznelson and Sack, 1966; Pittman and Pittman, 1966), but when so, the nonbutanol-extractable iodine has comprised a large fraction of the PBI. In other instances the PBI has been normal or low, but a large fraction of nonbutanol-extractable iodine (nBEI) has usually been present (Alexander and Burrow, 1970; DeGroot and Stanbury, 1959; Lizarralde et al., 1966). Recently, an abnormal thyroglobulin has been detected in a small goiter of a patient with no family history of thyroid disease (Kusakabe, 1972). This thyroglobulin was normal immunologically, electrophoretically, and in solubility and sedimentary characteristics, but the aromatic residues were not as accessable to iodination as normal. These findings suggested an abnormality in internal structure of the molecule.

An abnormal thyroglobulin found in the thyroids of South African cattle with congenital goiter differs from normal thyroglobulin in its ultracentrifugal pattern, salting-out curve, and electrophoretic mobility. Also, although it reacted with antithyroglobulin antibodies, the product did not precipitate. The ultracentrifugation pattern was also unlike that of thyroglobulin subunits (Robbins et al., 1966; van Zyl et al., 1969).

In Table 1 a summary is given of a number of studies which have been done on patients with familial goiter whose conditions have been variously diagnosed as "coupling defect," or characterized as having abnormalities of the thyroid iodoproteins, or as having iodoproteins in the peripheral blood. Only those patients are included in whom observations were made on blood and on thyroid tissue. Studies on sheep and cattle are also included. There are no studies of patients who cannot fit under the rubric, "errors in thyroglobulin synthesis."

Furth et al. (1970) studied a 35-year-old cretinous patient with goiter who had both thyroglobulin and an albumin-like iodoprotein in the thyroid. The iodine content of the thyroglobulin was 0.14 percent, which is low and surprisingly so since the thyroglobulin obtained 15 days after administration of radioiodine contained 12 percent labeled iodothyronines, and had a ratio of mono- to diiodotyrosine (MIT/DIT ratio) of less than 1. Unless the la-

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beled iodine was very far from equilibrium, it is difficult to interpret these findings, since usually when the iodine content of thyroglobulin is low the MIT/DIT ratio rises above 1 and the iodotyrosine-to-iodothyronine ratio is higher than reported in this patient. The investigators ventured the suggestion that their patient might have had an inability to hydrolyze the thyroglobulin. An alternative explanation might be that the basic defect was a diminished rate of synthesis of thyroglobulin coupled with an abnormal structure which made iodination or coupling difficult.

What criteria, then, are required for making a diagnosis of abnormal synthesis of thyroglobulin? Perhaps it is impossible to make this diagnosis with absolute security at present. Evidence favoring such an interpretation would be demonstration of a protein in the thyroid with many of the properties of thyroglobulin, but with other properties which are abnormal. It would be much more difficult to establish a limitation in the rate of synthesis of a normally structured thyroglobulin. Evidence favoring such a deduction might be the presence of a heavily iodinated thyroglobulin of normal structure and properties (but sharply reduced in concentration and quantity) together with other casually iodinated proteins, particularly albumin. A conclusion that a patient has a complete absence of any capacity to synthesize thyroglobulin or its subunits might follow the finding of only nonthyroglobulin-related iodinated protein in the thyroid, and absence of other well-defined defects in thyroid cell function.

In any study of patients of these kinds, one must be careful to guard against overinterpretation of changes in thyroid function which might be the result of intense tissue hyperplasia and hyperfunction caused by excessive TSH secretion. Since many of these patients secrete an iodinated albumin-like protein into the blood, there is perhaps no necessity at present for a separate designation for patients who are characterized by familial goiter and plasma iodoprotein. Current evidence does not establish such a distinct entity. Certainly there should be no such designation until a patient is found who clearly has no disorder or limitation of thyroglobulin synthesis, nor other defect of some other type, and yet secretes an iodoprotein into the blood.

Is a separate designation of "iodotyrosyl coupling defect" tenable? This designation would, of course, be applicable to patients synthesizing an abnormally structured thyroglobulin-like protein with a conformation which impaired coupling. Such patients would belong to a category of those described in the preceding paragraphs. To qualify for a diagnosis of "coupling defect" as a separate entity, it would be necessary to show that thyroglobulin of normal structure is formed in amounts adequate to act as substrate for iodination, and that its MIT and DIT content is normal in amount and ratio, but that, for such an amount and ratio, iodothyronine

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Table 1. Serum iodine values and thyroid soluble proteins in patients and in animals with apparent abnormalities in thyroid iodoprotein synthesis or with "coupling defect," or with the syndrome of familial goiter and abnormal serum iodoprotein

Age	Sex	PBI	NBEI (%)	Thyroglobulin (%)	Thyroid Soluble Proteins: Albumin or Albumin-like (%)	Reference
42	M	—	24.0	51.0[b,c]	49, S° 20, $w = 4$[b,c,d]	DeGroot and Stanbury, 1959
14	F	3.0	30.0	0[c,d]	S° 20, $w = 4$[a,b,c,d]	
35	M	9.0	4.7	>8.0[a]	42.0[c] } 58.0[a]	Pittman and Pittman, 1966
40	F	19.2	13.3	>8.0[a]	59.0[c] }	
35	?	9.3	9.3	41.0	69.0 (albumin and prealbumin[c])	Furth et al., 1970
35	M	4.5	57.0	10.0[a] >1.0[b] 0[c]	S° 20, $w = 4$[a,b,c,d] (largely prealbumin)	Lizarralde et al., 1966
46	M	4.2	2.8	10.0[a,b]	60.0[a,d]	Alexander and Burrow, 1970
5	M	4.9	2.2	±50.0[b,c]	±50.0[b,c] (Tg by electrophoresis was soluble in 1.9 M phosphate)	Stanbury et al., 1963
16	F	6.9	25.0	Trace	S° 20, $w = 4$[a,c,d,e]	Riddick et al., 1969
25	M	3.4	36.0	Trace	S° 20, $w = 3$–8[b,c,e]	Cassano, 1971
39	F	5.7	63.0	6.5[a,c]	59.1[a,b] (albumin-prealbumin[c])	Lissitzky et al., 1964
12	M	2.7	21.5	0[c,d,e]	45.0[e] 95.0[a,c,d]	Lissitzky et al., 1965
8	F	2.3	28.9	±10.0[d]	±90[b,c,d]	Lissitzky et al., 1968
15	F	1.6	0.3	?Trace[d]	Present[c,d,e]	Lissitzky et al., 1973
12	M	0.8	—	?Trace[d]		

PBI: Protein-bound iodine concentration of plasma.
NBEI: Nonbutanol-extractable iodine concentration of plasma.
[a] Identification by salting out.
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9	F	2.1	—	0[c]	Present[c]	Wiener and Lindeboom, 1964
12	F	2.0	—	0	Present[b,c,d]	Savoie et al.,
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	Sheep	5.7	59.7	Trace[d]	Present[a,c,e] S° 20, w = 3.2–7 and 8–9	Falconer et al., 1970
	Cattle	35.7	—	98, S° 20, w = 9 (immunoreactive for Tg)		van Zyl et al., 1969

concentration is low, and low for reasons other than rapid turnover. In addition, at the present level of understanding, it would probably be necessary to show that thyroglobulin prepared from such a patient is normal insofar as it can form iodothyronine at a normal rate in the presence of thyroid peroxidase. It is important to note that Lamas et al. (1972) report that peroxidase catalyzes the "coupling" reaction in the presence of thyroglobulin and a peroxide generating system, but without added iodide, iodothyronines were formed.

Since thyroglobulin as well as albumin will form thyroxine in the presence of iodide, a peroxide-generating system, and thyroid or other peroxidases, it is not at all clear that coupling in vivo has any requirements other than appropriate substrates (thyroglobulin and iodide) and the components of the iodide organification process of the gland (iodide, peroxide, peroxidase). If this is so, then normal organification would presuppose the conditions for coupling, apart from normality of substrate thyroglobulin. Thus, logically the conditions for a "coupling defect" seem to vanish. In any event, at present apparently there are no reports of any patients on whom a diagnosis of "iodotyrosyl coupling defect" has been rigorously established. In our own patients and perhaps in those of others, the findings, when critically examined, do not exclude the possibility of complete or nearly complete absence of thyroglobulin synthesis as the primary defect.

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OTHER INBORN ERRORS OF THE THYROID

Thyrotropin Deficiency

Two cretinous sisters without goiter have been described by Miyai et al. (1971). The parents were consanguine. ACTH and gonadotropins were normal, but no TSH was detected. There was no increase in plasma TSH after administration of thyrotropin-releasing hormone. A modest response in radioiodine uptake followed administration of TSH. Presumably, the pituitary was unable to synthesize thyrotropic hormone.

TSH Unresponsiveness

An 8-year-old cretin without goiter, but with consanguine parents, was unresponsive to either his own or administered TSH (Stanbury et al., 1968). The TSH concentration in the blood was high both by bioimmunoassay and radioimmunoassay. The thyroid showed enlarged cells with large irregular nuclei but almost no colloid. Thus, the gland appeared not to respond to TSH either by cell division or by synthesis of the thyroid-specific protein thyroglobulin. One might suggest an altered structure of the TSH receptor site on the thyroid cell surface as the cause of this disorder.

Elevated Thyroxine-Binding Globulin and Goiter

Shane et al. (1971) have described a large family with a high incidence of simple and nodular goiter and elevated plasma thyroxine-binding globulin (TBG). The trait for elevated TBG was transmitted as a sex-linked codominant. Genetic analysis of the large kindred indicated separate genetic loci for high TBG and goiter. In this family, as in others with genetically altered TBG, the binding abnormality has not been directly related to the presence of thyroid disease.

Familial Cretinism with Muscular Hypertrophy

Cross et al. (1968) have reported two sibs with cretinism and muscular hypertrophy of the Debre-Semelaigne type. Neither had a thyroid gland detectable by the usual methods but when one was given a large dose of labeled pertechnitate, a small thyroid gland was delineated in the neck in normal position. The nature of this defect is entirely unknown.

Thyroid Hormone Unresponsiveness

The precise way in which the thyroid hormones exert their actions on peripheral cells is still conjectural. Refetoff et al. (1967; 1972a,b) have extensively studied three members of a sibship who appear to have peripheral re-

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sistance to the action of the thyroid hormones. The findings among affected children included deafmutism, remarkable stippled epiphyses, small goiters, inconstant nystagmus, deafness, normal intellectual development, and sharp elevation in the plasma concentrations of thyroid hormones. The binding proteins were quite normal. With increasing doses of administered thyroxine or triiodothyronine, signs and symptoms of thyrotoxicosis developed in some systems, but not in others, although the doses required were considerably in excess of those usually effective. Entry of thyroid hormones into the cells was unimpeded. Bode et al. (1973) have studied an 8-year-old boy who also apparently had a reduced sensitivity of the peripheral tissues to the effects of thyroid hormones. The patient of Bode et al. (1973) was not deaf and did not have stippled epiphyses, but all had elevations in plasma hormone concentration and suppression of TSH action by administered thyroid hormone.

Familial Goiter with Intrathyroidal Calcification

Murray et al. (1966) reported this disorder in five generations of a single family. Enlargement of the thyroid appeared in the second decade of life. The central feature was a firm nodular goiter with dense spotty calcification. Search for the usual disorders of thyroid function was not rewarding. The patients were euthyroid. Histologically, in addition to follicles which appeared to be quite normal, there were dense fibrosis, calcific deposits, and nodule formation, together with areas of focal nonspecific thyroiditis. Results of chemical analysis for thyroid iodoamino acids were not significantly different from normal. No explanation for this disease has been offered.

SUMMARY

The most common phenotypic expression of the inborn errors of the thyroid system are goiter and hypothyroidism. Reduced synthesis, delivery, or effectiveness of thyroid hormones are met by increased secretion of thyrotropic hormone, which causes the thyroid to increase in size.

Phenotypic variation within the proposed groupings of the inborn thyroid errors can come about in at least three ways:

1. *Multiple alleles.* Different alleles may account for variable expression of the disorder among different families. For example, the peroxidase defect seems to be complete in some patients, in others the defect is present but not correctable by addition of hematin, and in still another it was corrected with hematin. This circumstance suggests three abnormal alleles for thyroid peroxidase, or possibly two abnormal alleles

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and a deletion at the same site. The available information on the disorders of thyroglobulin synthesis also suggests the probability of multiple alleles for a variety of abnormal gene products of the genetic loci coding for the subunits of thyroglobulin.

2. *More than one gene may be involved in a metabolic event.* The Pendred syndrome and the other types of peroxidase defect all share an impeded organification of iodine. It seems highly unlikely that the gene responsible for the Pendred syndrome is an allele of the genes causing the other forms. Dehalogenase deficiency only in the thyroid in some families, only in the periphery in others, and universally in still others suggests different genetic loci for isozymes. Indeed, the entire group of patients with familial goiter can be thought of as showing phenotypic variability under a chemical umbrella arising from different genetic defects which lead to a final common disturbance, which is impaired hormone supply with secondary gland hypertrophy.
3. *Environmental factors.* The expression of both the dehalogenase defect and the defect in iodide transport are dependent for their appearance on the available iodine in the diet. In both, supply of iodide in excess of normal daily needs will result in disappearance of the manifestations of the defects. Other environmental factors, such as protein nutrition (Ramalingaswami, 1965) may also condition the expression of thyroid disease.

The numbers of patients who have identifiable inherited defects in presently understood terms is small, but the numbers of patients with thyroid disorders with a genetic component is very large. The gene products which represent the inherited component of Graves' disease, of simple and nodular goiter, and of lymphocytic thyroiditis must be defined.

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4

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INTRODUCTION 82
DEFECTIVE MUCOPOLYSACCHARIDE CATABOLISM 83
Corrective Factors 84
From Corrective Factors to Lysosomal Enzyme Deficiencies 85
Unknown Enzymatic Deficiencies 89
Spectrum of Expression 91
The Hunter Heterozygote 92
DEFECTIVE UPTAKE OF LYSOSOMAL ENZYMES 92
Uptake of Lysosomal Enzymes in Vitro 93
Mucolipidoses: A Defect in Enzyme Uptake 94
IMPLICATIONS FOR ENZYME REPLACEMENT THERAPY 96
SUMMARY 97
REFERENCES 97

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Spectrum of Expression 91
The Hunter Heterozygote 92
DEFECTIVE UPTAKE OF LYSOSOMAL ENZYMES 92
Uptake of Lysosomal Enzymes in Vitro 93
Mucolipidoses: A Defect in Enzyme Uptake 94
IMPLICATIONS FOR ENZYME REPLACEMENT THERAPY 96
SUMMARY 97
REFERENCES 97

INTRODUCTION

The mucopolysaccharidoses have been among the most intriguing, as well as among the most baffling of genetic diseases. Recent discoveries have shown the biochemical principle underlying these disorders to be so simple that the reader may well wonder why its discovery was so long in coming.

Briefly, except for the Morquio syndrome, all the classified mucopolysaccharidoses (i.e., the Hurler, Scheie, Hunter, Sanfilippo, and Maroteaux-Lamy syndromes, and β-glucuronidase deficiency disease) involve a disturbance in the lysosomal catabolism of two polymeric substances, dermatan sulfate and heparan sulfate, singly or in combination. These polymers are made up of alternating residues of sulfated hexosamine (either glucosamine or galactosamine) and uronic acid (glucuronic or L-iduronic acid, the latter occasionally sulfated). The polymers may be quite large, with perhaps as many as 100 sugar residues per chain. The major degradative pathway is by lysosomal exoenzymes, that is, by glycosidases and sulfatases that cleave the chain from the nonreducing terminus and proceed sequentially, removing one sulfate group or one sugar residue at a time. Each chemically distinct group requires a different enzyme; thus the concerted action of over six enzymes may be required to degrade the chain. If any one of these enzymes is not functioning adequately, or is missing altogether, the degradative sequence is interrupted. It may then still be possible for some limited degradation to occur through the action of lysosomal endoglycosidases (i.e., enzymes such as hyaluronidase that can cleave the chain into fragments); however, further degradation of the fragments will also be blocked. Thus, in the normal situation, degradation of dermatan sulfate and heparan sulfate results in the formation of sulfate, which is excreted, and hexosamine and uronic acid which are recycled through the glycolytic pathway and hexose monophosphate shunt, respectively. By contrast, degradation of dermatan sulfate and heparan sulfate in mucopolysaccharidosis patients results in fragments; some of these are small enough to be released from cells and excreted in urine, whereas the remainder are retained intracellularly, within lysosomes. The storage is progressive, leading eventually to serious cellular pathology and, as a consequence, to clinical problems.

The principle underlying the mucopolysaccharidoses is thus identical to that responsible for the genetic glycosphingolipidoses, of which Gaucher's, Fabry's, and Tay-Sachs diseases are well-known examples. Glycosphingolipids that contain from one to seven sugars must be cleaved by lysosomal glycosidases specific for each sugar (for review, see Brady, 1973). Deficiency of any one enzyme interrupts the sequence, and the undegraded metabolite accumulates within lysosomes or is excreted in urine. This was first demonstrated by Brady et al. (1965) who associated the accumulation of glucosyl ceramide in Gaucher's disease with a deficit of glucosidase activity.

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Comparable enzyme defects have since been found in eight other glycosphingolipidoses.

But the glycosphingolipids are clearly defined and well characterized substances, whereas the polymers, dermatan sulfate and heparan sulfate, are heterogeneous; their detailed chemistry has only recently been investigated extensively. As a result, the elucidation of the mucopolysaccharidoses has not proceeded through the logical sequence that has characterized much of lipidosis research, i.e., chemical identification of the stored metabolite, discovery of an enzyme that cleaves the terminal residue of that metabolite, and comparison of the level of the enzymatic activity in extracts of tissues of patients and of normal persons. Instead, research into mucopolysaccharidoses proceeded primarily through analysis of sulfated mucopolysaccharide metabolism in intact cultured cells from normal persons and from affected patients, correction of the defect in the patients' cultured cells by secretions from cells of different genotype, purification of the "corrective factors," and identification of the factors as the missing lysosomal enzymes in the respective disorders.

Although lysosomal enzyme deficiencies are now known for most mucopolysaccharidoses, and can readily be surmised for the remainder, in this chapter we shall still take the reader through the circuitous route of the corrective factors. This is in part because of an obvious personal bias; but it is also because an unexpected dividend from the study of corrective factors has given an insight into an even more puzzling group of diseases, the multiple lysosomal enzyme deficiencies (I-cell disease and pseudo-Hurler polydystrophy, classified as mucolipidoses II and III, respectively). Study of these diseases has shown that there is not precise correspondence between lysosomal enzyme and corrective factor, but rather, that a corrective factor is a lysosomal enzyme with some special structural feature that allows it to enter cells efficiently. The concepts derived from the study of the mucolipidoses have obvious implications for enzyme replacement therapy in the mucopolysaccharidoses and other lysosomal disorders, as well as for understanding lysosomal physiology.

Clinical descriptions are omitted. A brief description of mucopolysaccharidoses and mucolipidoses has been presented in Volume VIII of this series (Scott, 1972). Extensive descriptions are available in reviews by Spranger and Wiedemann (1970), Spranger (1972), Dorfman and Matalon (1972), and in the monograph by McKusick (1972).

DEFECTIVE MUCOPOLYSACCHARIDE CATABOLISM

The concept of a disorder of mucopolysaccharide degradation originated in the electron microscopic studies of van Hoof and Hers (1964) who noted large vacuoles containing granular material in the hepatocytes of a Hurler

Comparable enzyme defects have since been found in eight other glycosphingolipidoses.

But the glycosphingolipids are clearly defined and well characterized substances, whereas the polymers, dermatan sulfate and heparan sulfate, are heterogeneous; their detailed chemistry has only recently been investigated extensively. As a result, the elucidation of the mucopolysaccharidoses has not proceeded through the logical sequence that has characterized much of lipidosis research, i.e., chemical identification of the stored metabolite, discovery of an enzyme that cleaves the terminal residue of that metabolite, and comparison of the level of the enzymatic activity in extracts of tissues of patients and of normal persons. Instead, research into mucopolysaccharidoses proceeded primarily through analysis of sulfated mucopolysaccharide metabolism in intact cultured cells from normal persons and from affected patients, correction of the defect in the patients' cultured cells by secretions from cells of different genotype, purification of the "corrective factors," and identification of the factors as the missing lysosomal enzymes in the respective disorders.

Although lysosomal enzyme deficiencies are now known for most mucopolysaccharidoses, and can readily be surmised for the remainder, in this chapter we shall still take the reader through the circuitous route of the corrective factors. This is in part because of an obvious personal bias; but it is also because an unexpected dividend from the study of corrective factors has given an insight into an even more puzzling group of diseases, the multiple lysosomal enzyme deficiencies (I-cell disease and pseudo-Hurler polydystrophy, classified as mucolipidoses II and III, respectively). Study of these diseases has shown that there is not precise correspondence between lysosomal enzyme and corrective factor, but rather, that a corrective factor is a lysosomal enzyme with some special structural feature that allows it to enter cells efficiently. The concepts derived from the study of the mucolipidoses have obvious implications for enzyme replacement therapy in the mucopolysaccharidoses and other lysosomal disorders, as well as for understanding lysosomal physiology.

Clinical descriptions are omitted. A brief description of mucopolysaccharidoses and mucolipidoses has been presented in Volume VIII of this series (Scott, 1972). Extensive descriptions are available in reviews by Spranger and Wiedemann (1970), Spranger (1972), Dorfman and Matalon (1972), and in the monograph by McKusick (1972).

DEFECTIVE MUCOPOLYSACCHARIDE CATABOLISM

The concept of a disorder of mucopolysaccharide degradation originated in the electron microscopic studies of van Hoof and Hers (1964) who noted large vacuoles containing granular material in the hepatocytes of a Hurler

patient. They interpreted these vacuoles to be lysosomes abnormally distended with undigested mucopolysaccharide. The observation followed the discovery in Pompe's disease (Baudhuin et al., 1964) of lysosomal storage of glycogen associated with the absence of a lysosomal enzyme, acid maltase; this led van Hoof and Hers to speculate on the possibility of an analogous defect in the Hurler syndrome. However, since there was no experimental evidence, they were unable to rule out the equally plausible hypothesis of overproduction of mucopolysaccharide, with secondary sequestration of excess material in lysosomes.

The question was only resolved a few years later, when the techniques of cell culture were applied to the problem. As Danes and Bearn (1965, 1966) first showed, fibroblasts cultured from the skin of Hurler and Hunter patients have mucopolysaccharide accumulation similar to that in the patients' tissues (Matalon and Dorfman, 1969). Cell culture made possible the type of experiment that could not be performed with an occasional biopsy or autopsy specimen. Using $^{35}SO_4$ as marker for sulfated mucopolysaccharide, Fratantoni et al. (1968a) showed that the difference in the kinetics of mucopolysaccharide turnover between fibroblasts derived from normal persons and those derived from Hurler and Hunter patients could not be ascribed to overproduction or diminished secretion in the patient's cells, but were compatible with a reduced rate of mucopolysaccharide degradation (for a detailed discussion of the kinetic experiments, see Neufeld and Cantz, 1973).

Corrective Factors

Although the Hurler and Hunter syndromes are genetically distinct, the former being inherited in autosomal recessive and the latter in X-linked recessive manner, fibroblasts from the two types of patients could not be distinguished by simple observation of mucopolysaccharide accumulation or turnover. However, Fratantoni et al. (1968b, 1969) found that mucopolysaccharide catabolism became normal when the two types of fibroblasts were mixed with each other. Cell contact was found not necessary for the correction, since medium preincubated with Hunter fibroblasts could accelerate mucopolysaccharide catabolism in Hurler fibroblasts, and vice versa. Furthermore, cross-correction was not limited to these two genotypes. Hunter cells, for example, could be corrected by the addition of medium preincubated with normal cells, or cells from any genotype other than Hunter. The defective mucopolysaccharide catabolism of Hunter cells thus seemed to be a consequence of a deficit of a substance required for mucopolysaccharide catabolism, that could be produced by cells of other genotype, and that could be administered exogenously to Hunter cells with corrective results. That substance was designated the "Hunter corrective factor." Analogous but distinct corrective factors were found for other

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mucopolysaccharidoses, and were named in each case for the genotype that they corrected. A quantitative assay was developed for corrective activity, and the purification of the factors was undertaken from normal urine—a source that was found as potent as fibroblast secretions and, of course, more readily available (Neufeld and Cantz, 1971).

Correction, whether by means of cell mixing, or by addition of conditioned medium or purified factors, proved a powerful tool for classifying the mucopolysaccharidoses. The biochemical classification followed the original clinical classification of McKusick (1966) in broad outline, but differed in some significant aspects. Cells from all Hunter patients tested have a deficiency of the same factor, which is specific for that disorder (Cantz, et al., 1972). Cells from Hurler patients have a deficit of a different factor, distinct from the deficits of the Hunter, Sanfilippo, and Maroteaux-Lamy syndromes, but shared by cells from patients with the Scheie syndrome (Barton and Neufeld, 1971). This was very surprising because the Hurler syndrome is the most severe of the mucopolysaccharidoses, whereas the Scheie syndrome is the most benign. Another finding was that of biochemical heterogeneity in the Sanfilippo syndrome, which turned out to be divisible into two groups, arbitrarily designated A and B, each with its own factor deficiency (Kresse et al., 1971). The Maroteaux-Lamy syndrome has its own specific and unique factor deficiency (Barton and Neufeld, 1972).

From Corrective Factors to Lysosomal Enzyme Deficiencies

When correction was discovered, we thought that this phenomenon might be akin to bacterial transformation by nucleic acids. However, it soon became evident that the factors behaved as proteins, and that correction was probably a matter of replacing the enzyme missing in the disorder. That hypothesis was strengthened by the finding of Wiesmann et al. (1971b) and of Porter et al. (1971) that arylsulfatase A could correct the defect in cells of patients with metachromatic leukodystrophy (which are defective in that enzyme) in a manner analogous to correction of the defect in fibroblasts from mucopolysaccharidosis patients by the corrective factors.

Strong support for considering the corrective factors as likely candidates for the "missing enzymes" came from study of an atypical patient whose clinical development was reminiscent of that of a Hurler or a Hunter patient, but whose cells were not deficient in the Hurler or Hunter factors (Sly et al., 1973). Rather, they were found deficient in the lysosomal enzyme, β-glucuronidase. Bovine β-glucuronidase functioned as corrective factor to decrease mucopolysaccharide accumulation in those cells. Identity of corrective factor activity and enzymatic activity was demonstrated by coelectrophoresis of the two in polyacrylamide gel (Hall et al., 1973).

How could one assign an enzymatic function to the corrective factors?

Purified Sanfilippo A, Hurler, and Hunter factors could not be equated with any of the lysosomal glycosidases known at the time. We reasoned that if a corrective factor were a lysosomal hydrolase, then the mucopolysaccharide accumulated in the corresponding uncorrected fibroblasts would be its substrate. If one labeled the mucopolysaccharide by administering the appropriate isotope to uncorrected cells, isolated the mucopolysaccharide and incubated it with the corrective factor, one might observe degradation products.

This approach proved successful with the Sanfilippo A system (Kresse and Neufeld, 1972). The mucopolysaccharide accumulated by Sanfilippo A fibroblasts (heparan sulfate) was labeled with $^{35}SO_4$ and incubated with highly purified (though not pure) Sanfilippo A factor. Sulfate was released, implicating the enzyme as a sulfatase for heparan sulfate. Preliminary evidence suggested that of the several kinds of sulfate groups encountered in heparan sulfate, the ones that served as substrate for the Sanfilippo A corrective factor were those linked to the amino group of glucosamine (referred to as N-sulfate, or more properly, sulfamate residues).

The same approach was used for identifying the function of the Hunter corrective factor (Bach et al., 1973). Mucopolysaccharide (dermatan sulfate and heparan sulfate) was labeled in sulfate, hexosamine, or uronic acid residues by incubating uncorrected Hunter fibroblasts in isotopic precursors: $^{35}SO_4$, [^{3}H]glucosamine and [^{3}H]galactose, respectively. The labeled mucopolysaccharide was isolated and incubated with Hunter factor. The factor catalyzed the release of some radioactivity from the [^{35}S] but not from the [^{3}H]-labeled mucopolysaccharide. The radioactivity was released in the form of inorganic sulfate, thus implicating the Hunter factor as a sulfatase. The amount of radioactive sulfate released was so small (about 2 percent of the radioactive polymer) as to make us wonder at first about its significance. However, this is the amount of sulfate one might expect to be released from a chain containing 50 sulfated carbohydrate residues, of which only the terminal one is a substrate.

The particular type of sulfate group that serves as substrate for the Hunter corrective factor is that which is linked to L-iduronic acid. This has been demonstrated in several ways, of which the most direct involved the use of the disaccharide 4-0-α-L-sulfoiduronosyl-D-sulfoanhydromannose (Fig. 1). The combined action of the Hunter factor and of α-L-iduronidase released iduronic acid from the disaccharide, though neither enzyme alone catalyzed such release. Direct analysis has shown the presence of sulfated uronic acid at the nonreducing end of the dermatan sulfate that accumulates in Hunter fibroblasts (Sjöberg et al., 1973).

Fibroblasts from Hunter patients are specifically deficient in sulfoiduronate sulfatase; the methods used, however, are too cumbersome for routine diagnostic purposes. Until simpler methods can be worked out,

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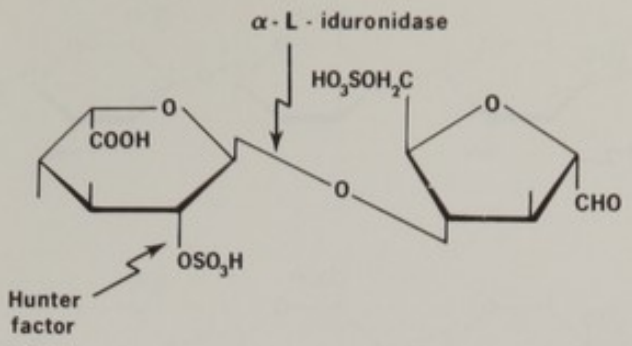


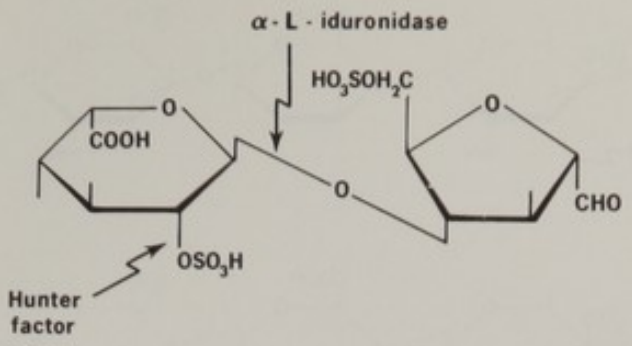
Fig. 1. Structure of 4-0-α-L-sulfoiduronosyl-D-sulfoanhydro-mannose, the disaccharide used to demonstrate the enzymatic function of the Hunter corrective factor.

measurement of [^{35}S]mucopolysaccharide accumulation and correction remains the method of choice for the biochemical diagnosis of the Hunter syndrome.

The defect in the Hurler syndrome was elucidated by the efforts of three laboratories, using different but convergent approaches. Matalon, et al. (1971), noting that dermatan sulfate and heparan sulfate had in common the presence of α-L-iduronosyl residues for which no degradative enzyme was known, proceeded to search for a hydrolase for that linkage. They found α-L-iduronidase activity in extracts of fibroblasts and liver and in the urine of normal persons, but a deficiency of that activity in analogous material derived from Hurler patients (Matalon and Dorfman, 1972).

By a complicated organic procedure, Friedman and Weissman (1972) synthesized phenyl α-L-iduronide, with which Weissmann and Santiago (1972) established the presence of a specific α-L-iduronidase in lysosomes of rat liver. Using the same synthetic substrate, Bach et al. (1972b) established the identity of Hurler corrective factor with α-L-iduronidase. Fibroblasts from Hurler or Scheie patients, previously shown associated with a deficiency of Hurler corrective factor, were found totally deficient in α-L-iduronidase. Fibroblasts of heterozygotes have an intermediate level of enzyme (Bach et al., 1972a).

Elucidation of the defect of the Sanfilippo B syndrome, like that of the Hurler syndrome, also involved independent approaches in different laboratories. Because heparan sulfate contains N-acetyl α-glucosaminide linkages, O'Brien (1972) examined fibroblasts and tissues of Sanfilippo patients for N-acetyl α-glucosaminidase. He found a marked deficit of that enzyme in material originating from patients that had been classified as Sanfilippo B by correction tests and a partial deficiency in fibroblasts from their parents. Von Figura and Kresse (1972) on the other hand, purified the Sanfilippo B factor and identified it as the enzyme N-acetyl-α-glucosaminidase.



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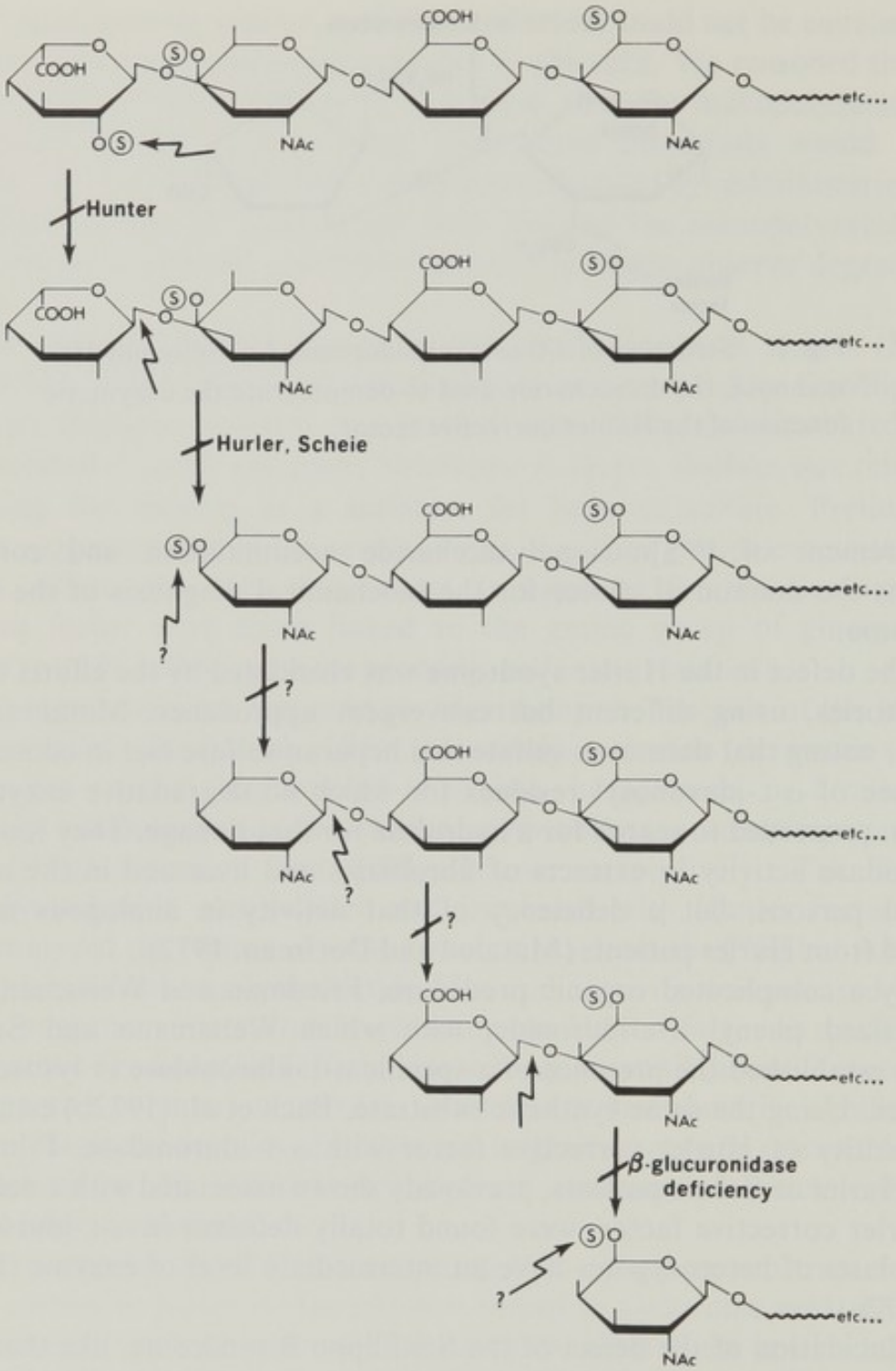
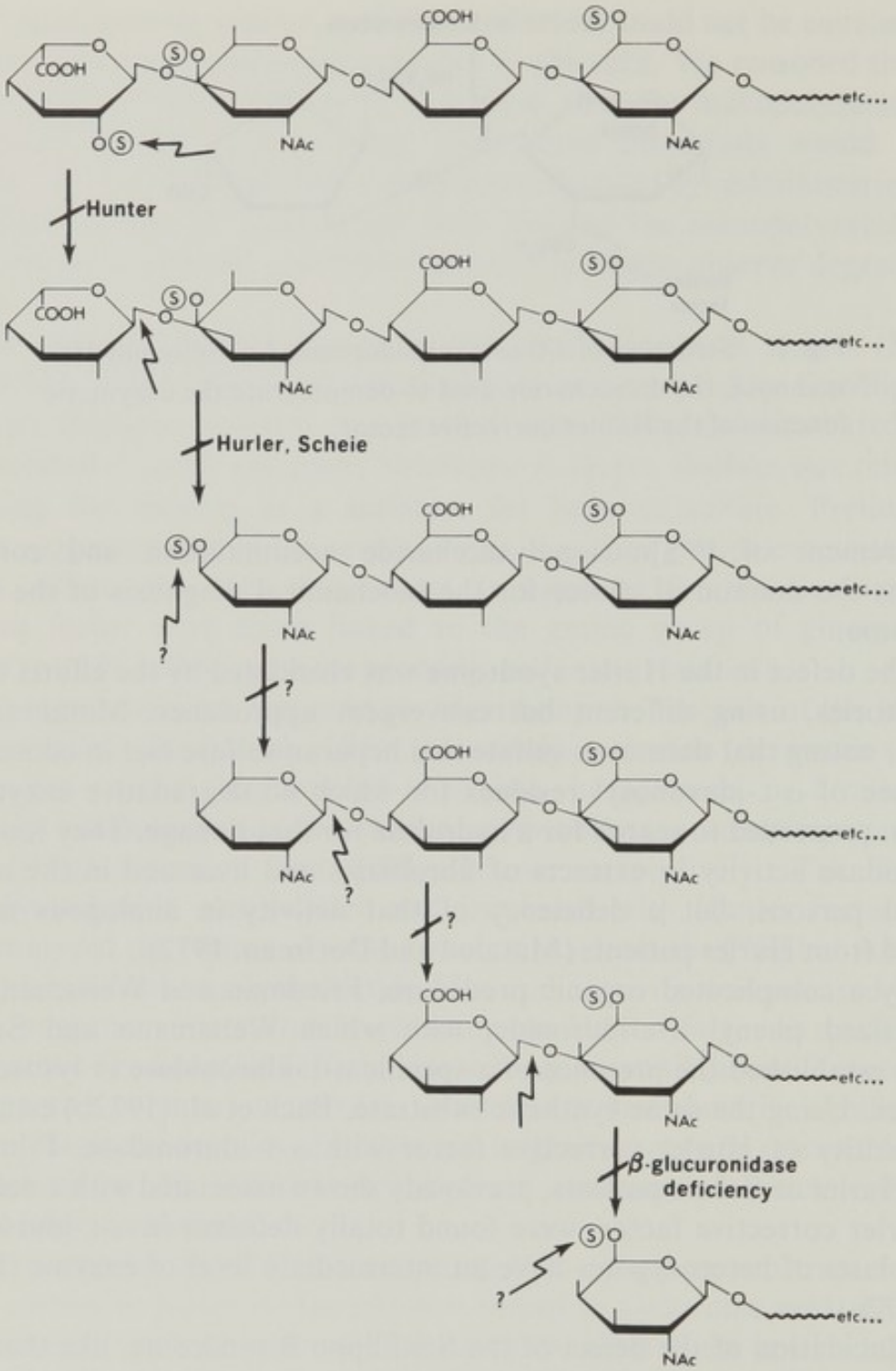


Fig. 2. Schematic representation of the catabolism of dermatan sulfate and of enzymes and enzyme deficiency diseases that are known or postulated to occur along that pathway. Every residue of dermatan sulfate is shown once; this should not be construed to mean that these residues occur in equivalent amounts in the chain (for discussion of the irregularity and heterogeneity of dermatan sulfate, see Neufeld and Barton, 1972).



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Unknown Enzymatic Deficiencies

The degradation of dermatan sulfate and of heparan sulfate, as well as the enzymatic blocks discussed above, are shown schematically in Figures 2 and 3. Note that there are still several linkages in search of an enzyme and of a disease: two in dermatan sulfate and two in heparan sulfate, involving in each case the sulfated hexosamine residues.

The enzyme defect in the Maroteaux-Lamy syndrome is not yet elucidated; since only dermatan sulfate is excreted in that disease, one would suspect that the deficit would be of an enzyme needed for the degradation of dermatan sulfate, but not heparan sulfate, that is, for the removal of sulfated N-acetyl-β-hexosaminide residues, shown on the third and fourth rows of Figure 2. Recently, a deficiency of arylsulfatase B has been noted in tissues and fibroblasts of Maroteaux-Lamy patients (Austin, 1973; Kihara et al. 1973); the relationship of that enzyme to mucopolysaccharide degradation is not known, nor its relationship to the Maroteaux-Lamy corrective factor. Experience with β-galactosidase deficiency in the Hurler, Hunter, and Sanfilippo syndromes (van Hoof and Hers, 1968; Öckerman, 1968; Gerich, 1969; MacBrinn et al., 1969; Ho and O'Brien, 1969) should alert us to possible enzyme deficiencies that are secondary to the primary defect.

Enzymes catalyzing the unknown steps in the degradation of heparan sulfate (third and seventh rows of Fig. 3) can reasonably be postulated to exist, and their deficiency should result in as yet unknown biochemical subtypes of the Sanfilippo syndromes.

Dermatan sulfate and heparan sulfate contain the same uronic acids (L-iduronic acid, sulfated or unsulfated, and glucuronic acid), hence enzymatic defects involving uronic acid residues should result in storage and excretion of both polymers. This is generally the case; most Hurler and Hunter patients excrete both dermatan sulfate and heparan sulfate. A few such patients may excrete only one of those polymers; this can be explained by assuming that the hydrolase, altered by mutation, retains activity toward one substrate but not the other. The correlation of enzyme deficit and urinary excretion of mucopolysaccharide is more puzzling in the case of β-glucuronidase deficiency, since β-glucuronosyl linkages (in contrast to α-L-iduronosyl linkages) are not limited to dermatan sulfate and heparan sulfate, but are common to the other major mucopolysaccharides: chondroitin 4- and 6-sulfates and hyaluronic acid. One would expect patients with β-glucuronidase deficiency to excrete fragments of all these mucopolysaccharides. Yet the urine of one β-glucuronidase-deficient patient contains excess chondroitin 4- and 6-sulfates (Sly et al., 1973), and that of another excess undegraded dermatan sulfate (Beaudet et al., 1973). Again, one must postulate the existence of an altered enzyme with activity for some polymers but not for others. A case of "chondroitinsulfaturia" with normal level of β-glucuronidase activity may represent a novel mucopolysaccharidosis (Spranger et al., 1971).

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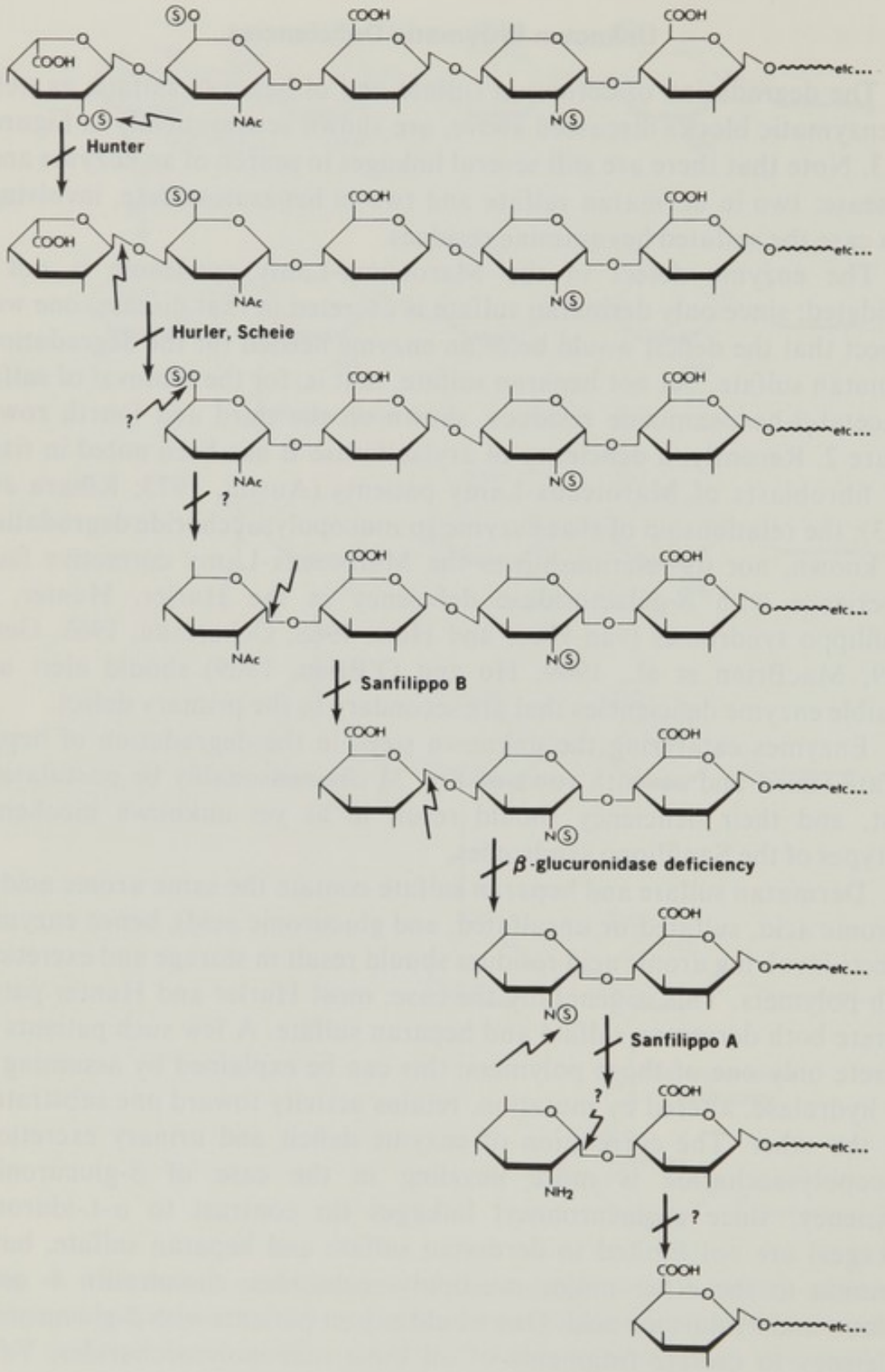
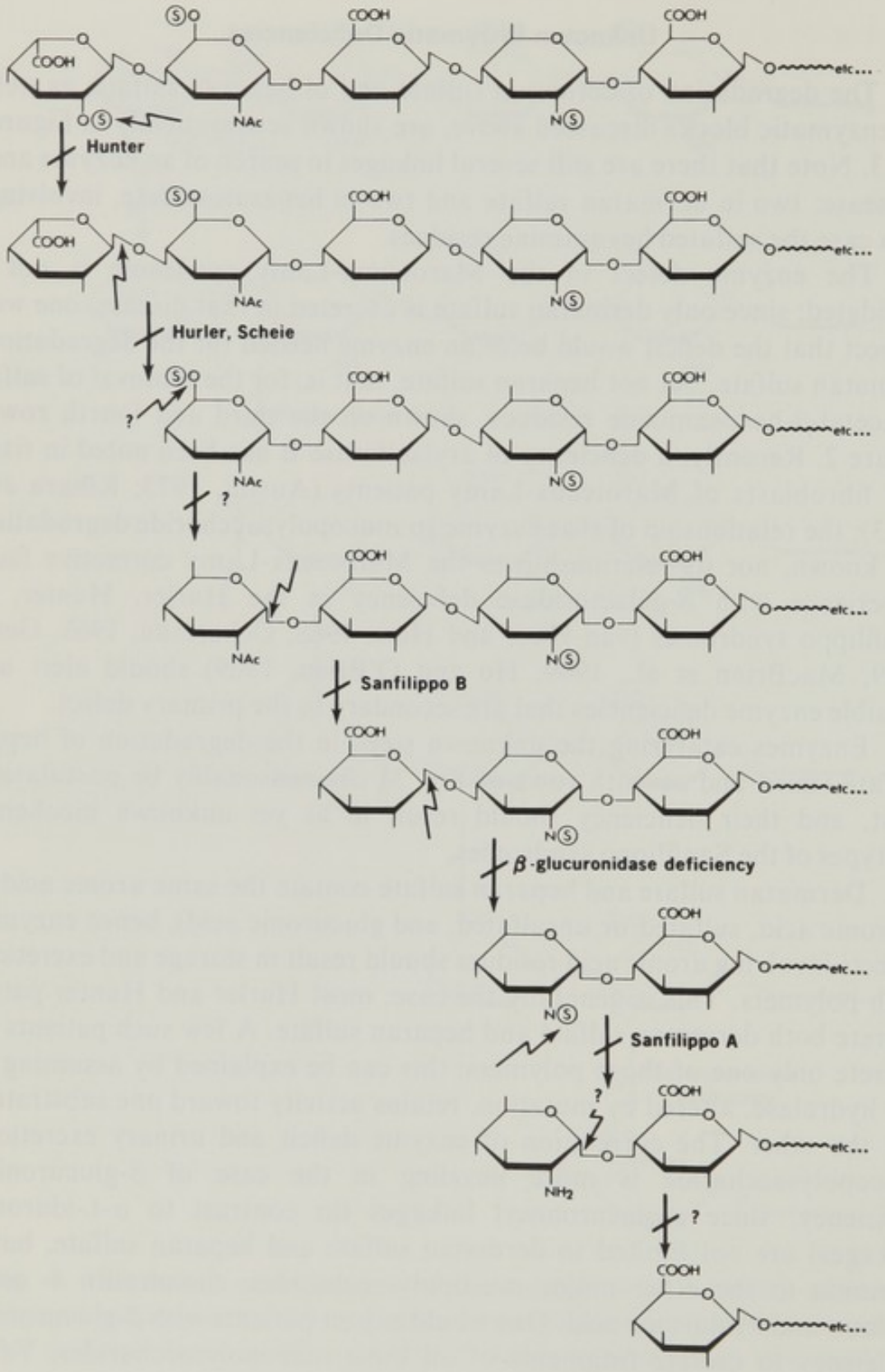


Fig. 3. Same representation as Fig. 2, but for heparan sulfate. The structure of heparan sulfate is known but imprecisely, and the diagram above is modeled on the structure of heparin, a related compound for which considerably more information is available (for review, see Neufeld and Barton, 1972).



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The Morquio syndrome, associated with excretion of keratan sulfate, has not yet been studied successfully in cultured skin fibroblasts, perhaps because skin fibroblasts do not produce keratan sulfate. In the absence of experimental evidence, one should be careful not to classify this disease as a disturbance of keratan sulfate catabolism, simply on the basis of analogy with other mucopolysaccharidoses. Since keratan sulfate occurs in cartilage and cornea, and since these tissues are affected in the Morquio syndrome, the study of cultured chondrocytes or corneal fibroblasts will no doubt be necessary to elucidate the defect in that disorder.

Spectrum of Expression

An intriguing problem in the mucopolysaccharidoses is the existence of mild and severe disorders characterized by the same enzyme deficiency. For example, in spite of the major clinical differences between Hurler and Scheie patients, fibroblasts from either are devoid of α-L-iduronidase activity and accumulate similar levels of [^{35}S]mucopolysaccharide. It has been postulated that the Hurler and Scheie mutations are not identical but allelic, the latter resulting in modified α-L-iduronidase with some residual activity in vivo, (even though it has yet to be demonstrated in vitro). McKusick et al. (1972) pointed out that there should exist "genetic compounds" with one Hurler gene and one Scheie gene. Patients with a phenotype intermediate between Hurler and Scheie and with iduronidase deficiency are not uncommon, and apparently represent the presumed compounds. There is no theoretical reason to believe that the number of alleles is restricted to two; information on hemoglobinopathies and glucose 6-phosphate dehydrogenase (G-6-PD) deficiencies indicates that many different allelic mutations arose in different parts of the world. Multiplicity of alleles would imply the existence of numerous compound forms as well.

Mild and severe variants of the Hunter syndrome share a deficiency of the Hunter corrective factor (sulfoiduronate sulfatase) (Cantz et al., 1972). Again, two or more alleles are postulated, some leading to an enzyme with residual activity in vivo, and others to no activity at all. There are also mild and severe variants of the Maroteaux-Lamy syndrome, characterized by a deficiency of the same corrective factor. Interestingly, the severe form of the Maroteaux-Lamy syndrome is clinically similar to the intermediate form of α-L-iduronidase deficiency, whereas the milder form is reminiscent of the Scheie syndromes (McKusick et al., 1972).

The postulate of allelism and genetic compounds must eventually be proved rigorously. This shall be done when it becomes technically feasible to isolate modified iduronidase proteins, with or without residual catalytic activity.

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that in cell culture, 90 percent correction can be achieved by introducing enough α-L-iduronidase into Hurler fibroblasts to bring their activity to 10 percent of normal. A more striking example is seen in the case of homocystinuria caused by cystathionine synthase deficiency. Administration of massive doses of pyridoxine to some patients with this disorder increases their level of cystathionine synthase activity from 1 percent to 4 percent of the normal. This small enhancement is sufficient to reverse the biochemical manifestations of the disease (Mudd, 1971).

The Hunter Heterozygote

Because of the X-linked inheritance of the Hunter syndrome, heterozygotes are, in accord with the Lyon hypothesis, mosaics of normal and Hunter cells. This was demonstrated by Danes and Bearn (1967) who, on cloning cultures of heterozygotes, found the two expected populations. The apparent paradox presented by the normal phenotype of these carriers, half of whose cells should be storing mucopolysaccharide, led Fratantoni et al. (1968b) to postulate interaction of normal and abnormal cells. In present concepts, normal cells supply sulfoiduronate sulfatase and thereby correct the Hunter cells.

Occasionally, only one population of cells is observed in culture of heterozygotes, presumably because of some selective advantage. We had assumed such selection in vitro to be random, although only the selection of Hunter cells would be readily observable (since the starting culture of mixed cells, and a selected population of normal cells would not be distinguishable from each other without cloning techniques). However, Booth and Nadler (1973) report a consistent selection of Hunter cells in culture, particularly on thawing heterozygous strains that had been stored in liquid nitrogen.

In rare instances, such selection may also occur in vivo. Milunsky and Neufeld (1973) described a severely affected girl, whose fibroblasts (as well as those of her affected brother) had all the biochemical manifestations of the Hunter syndrome, including a deficiency of sulfoiduronate sulfatase. The normal phenotype of her father and her 46,XX karyotype strongly suggest that she is a heterozygote, in whom those cells that have an active maternal X chromosome (bearing the Hunter gene) had been selected, presumably because of some unrelated deleterious gene on the paternal X chromosome. However, the possibility of a new mutation on the paternal chromosome, though unlikely, cannot be ruled out (Punnett, 1973).

DEFECTIVE UPTAKE OF LYSOSOMAL ENZYMES

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located, so that the degradative pathway is reconstituted. Uptake of α-L-iduronidase (Bach et al., 1972b), N-acetyl-α-glucosaminidase (von Figura and Kresse, 1973), and β-glucuronidase (Hall et al., 1973) accompany the correction of Hurler cells, Sanfilippo B cells, and β-glucuronidase deficient cells, respectively.

Uptake of Lysosomal Enzymes in Vitro

We originally envisioned entry as occurring by fluid endocytosis (Neufeld and Barton, 1972)—that is, a process by which the cell indiscriminately imbibes some medium, with all its soluble and particulate contents (Jacques, 1969). However, the astonishing efficiency of uptake of α-L-iduronidase (nearly half the activity supplied in the medium, in some experiments), in contrast to the minute uptake of ^{125}I-albumin, suggested a more selective process (Bach et al., 1972b). Adsorptive pinocytosis, requiring binding of the protein to sites on the cell surface, is a process that would supply the required specificity. The lysosomal enzyme would be recognized because the binding site has affinity for some structural feature on the protein. Subsequent steps would be invagination of the membrane, pinching off of the pinocytic vesicle (with hydrolases attached to what is now the inner surface), and fusion of the pinocytic vesicle with a preexisting lysosome.

In concept, the uptake and sequestration of hydrolases into fibroblast lysosomes would be analogous to the uptake and sequestration of certain plasma proteins into lysosomes of rat hepatocytes in vivo (Gregoriades et al., 1970; Morell et al., 1971; Van Lenten and Ashwell, 1972). The latter process requires that the plasma proteins in question, all of which are glycoproteins, have exposed β-galactosyl termini. Recognition of hydrolases by fibroblasts probably also requires a carbohydrate marker (though probably not galactose). Evidence for this is found in studies of the uptake of N-acetyl-β-hexosaminidase into fibroblasts (Hickman et al, 1974); uptake of this enzyme (derived from normal fibroblast secretions) is markedly diminished if the secretions are first treated with periodate under conditions chosen for the selective destruction of carbohydrate residues. Though such experiments have yet to be performed with those enzymes that act as corrective factors, it seems plausible that the same principle would apply.

Mucolipidoses: A Defect in Enzyme Uptake in Vivo

Fibroblasts of patients with mucolipidosis II (inclusion-cell, or I-cell, disease) do not cross-correct with those of Hurler and Hunter patients (Neufeld and Cantz, 1971). It was therefore very surprising to find that these fibroblasts, or "I-cells", secreted essentially normal amounts of α-L-iduronidase. Careful quantitation showed that, relative to catalytic activity, the α-L-iduronidase of I-cells had but one-seventh the corrective activity of the corre-

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sponding normal enzyme (Hickman and Neufeld, 1972). The difference was even greater in β-glucuronidase, the enzyme from I-cell secretions having less than one-tenth the corrective activity of its normal counterpart.

Since correction includes uptake of the enzyme from the medium in addition to enzymatic function within the recipient cells, Hickman and Neufeld (1972) postulated that the hydrolases secreted by I-cells were somehow defective so that they were not taken up from the medium. Direct measurement of uptake showed that, indeed, only one-eighth as much of the N-acetyl-β-hexosaminidase secreted from I-cells would enter into fibroblasts as would the N-acetyl-β-hexosaminidase secreted from normal fibroblasts. (The uptake of that particular enzyme into cells deficient in N-acetyl-β-hexosaminidase was a system chosen for study because of technical simplicity).

Inclusion cells are so named because they contain lysosomes so large and so packed with debris that they are visible by phase microscopy in the form of black inclusions (Hanai et al., 1971). There is a deficit of nearly all lysosomal enzymes, acid phosphatase and β-glucosidase being the two recorded exceptions (e.g., Leroy et al., 1972). Some enzymes (β-galactosidase and α-L-fucosidase) may be totally missing, whereas others, such as α-L-iduronidase, β-glucuronidase, and N-acetyl-β-hexosaminidase may be present to the extent of 5 to 30 percent of the normal level. Yet, the primary defect cannot be in the synthesis of these enzymes, since several of them have been found to be normal or elevated in the culture medium (Wiesmann et al., 1971a). The patients have an enormous elevation of some of these enzymes in the plasma, cerebrospinal fluid, and urine (Wiesmann et al., 1971c); an affected fetus has a smaller excess in the surrounding amniotic fluid (Huijing et al., 1973).

I-cell disease may thus be considered as a condition in which hydrolases are extracellular instead of within lysosomes. Coupled to the information that hydrolases secreted by I-cells do not enter fibroblasts, this forces us to the startling conclusion that the only mechanism for packaging hydrolases into lysosomes of fibroblasts must be by secretion from the cell and uptake into neighboring cells by some selective pinocytosis. In I-cell disease the defective hydrolases would not be taken up as efficiently and would therefore remain in the extracellular fluid (Fig. 4). The primary defect in I-cell disease would be in the formation of the recognition marker (carbohydrate?) needed for uptake. Since such a marker might be composed of a chain of several sugars, a defect in one of several enzymes might lead to the same result. Genetic heterogeneity in this disorder is therefore anticipated.

The above hypothesis of lysosomal enzyme packaging by way of secretion and selective uptake is at variance with the accepted concept of a primary lysosome containing all the hydrolases, derived by a completely intracellular pathway from the Golgi apparatus or endoplasmic reticulum, and eventually merging with a pinocytic vesicle that contains no enzymes

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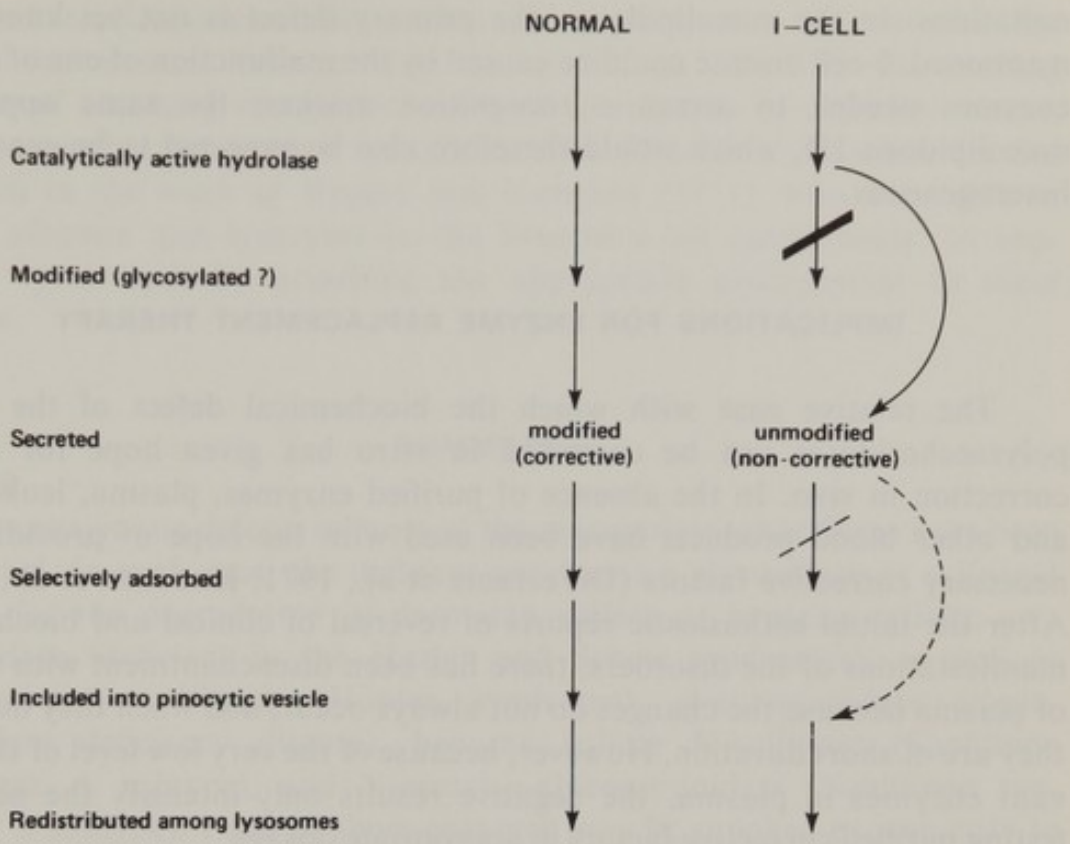


Fig. 4. Postulated pathway of lysosomal enzyme packaging in fibroblasts, by way of secretion and selective uptake. In I-cell disease as well as in pseudo-Hurler polydystrophy, the primary defect would be in the modification of the hydrolase; the unmodified hydrolase would not be taken up effectively and as a result would accumulate extracellularly.

(DeDuve and Wattiaux, 1966). However, that concept was derived primarily from studies of hepatocytes, neurons and macrophages; electron microscopy shows that I-cell disease affects cells of connective tissue and a few other cell types, whereas neurons and hepatocytes are spared (for review, see Tondeur and Neufeld, 1973). Perhaps those cells that are affected by the I-cell mutation are just those that have only the extracellular pathway of lysosomal enzyme packaging.

Though studies on pseudo-Hurler polydystrophy (mucolipidosis III) are still scanty, there is good evidence that this disorder is biochemically related to I-cell disease. Plasma and urine of patients have an excess of certain lysosomal hydrolases, whereas cultured fibroblasts have a marked deficiency (Thomas et al., 1973; Glaser et al, 1974). The N-acetyl-β-hexosaminidase from secretions of mucolipidosis III fibroblasts likewise fails to be taken up (Hickman et al., 1974). But mucolipidosis III is a mild disease, whereas I-cell disease is perhaps even more severe than the Hurler syndrome. However, it is incorrect to conclude, by analogy with the severe and mild variants of mucopolysaccharidoses, that mucolipidoses II and III represent allelic

mutations. In the mucolipidoses, the primary defect is not yet known. As mentioned, I-cell disease could be caused by the malfunction of one of several enzymes needed to attach a recognition marker; the same applies to mucolipidosis III, which would therefore also be expected to be genetically heterogeneous.

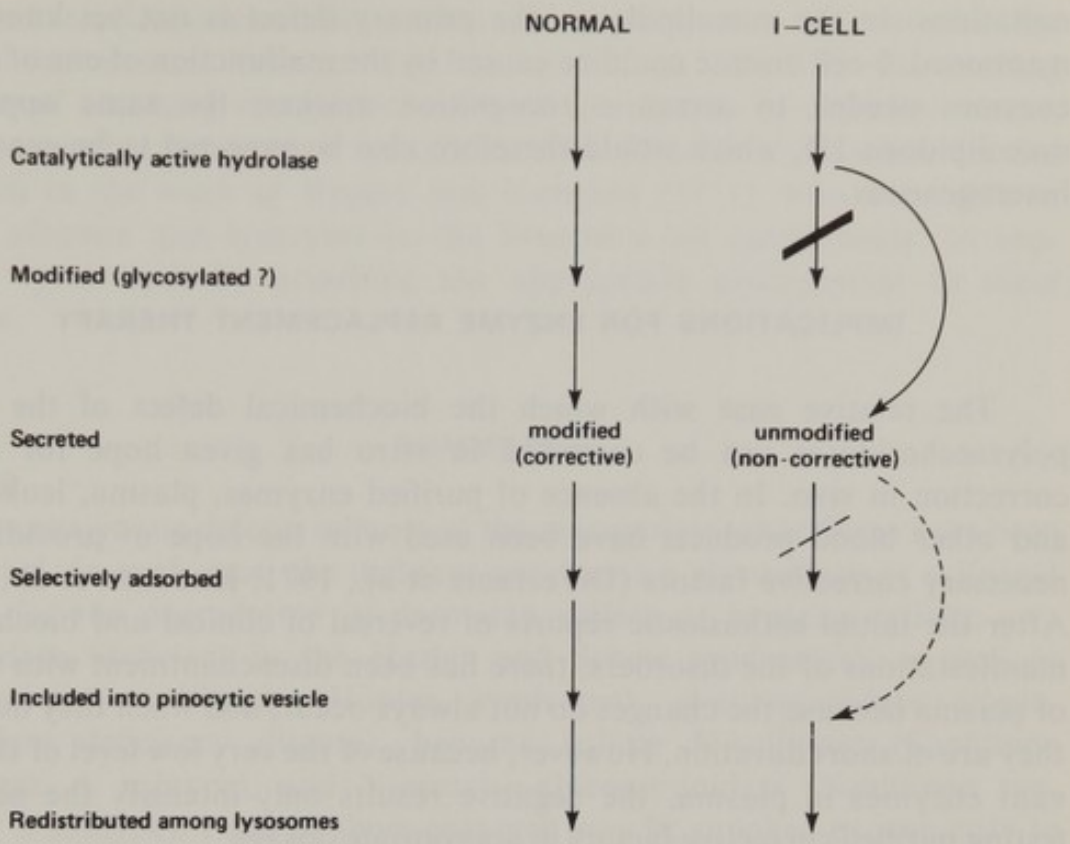
IMPLICATIONS FOR ENZYME REPLACEMENT THERAPY

The relative ease with which the biochemical defect of the mucopolysaccharidoses can be corrected in vitro has given hope for similar correction in vivo. In the absence of purified enzymes, plasma, leukocytes, and other blood products have been used with the hope of providing the necessary corrective factors (DiFerrante et al., 1971; Knudson et al., 1971). After the initial enthusiastic reports of reversal of clinical and biochemical manifestations of the disorders, there has been disenchantment with the use of plasma because the changes do not always occur, and when they do occur they are of short duration. However, because of the very low level of the relevant enzymes in plasma, the negative results only intensify the need for testing purified corrective factors in appropriate dosage.

Replacement of lysosomal enzymes in genetic disease was proposed nearly a decade ago by Baudhuin et al. (1964) on the assumption that pinocytosis would ensure delivery of an injected enzyme to the interior of a lysosome. However, injection of a potent fungal α-glucosidase into a child with Pompe's disease did not result in the removal of lysosomal glycogen deposits in hepatocytes within the 6 hr intervening between the injection and the patient's death. A similar experiment was repeated by Hug and Schubert (1967); in this case, clearing of lysosomal glycogen was seen after treatment for 18 days.

In retrospect, it is unfortunate that the failure of the original experiment was not given more attention. For it is now apparent that its theoretical basis was only partly correct. Nonspecific pinocytosis of proteins does occur, but very inefficiently. Selective, efficient uptake requires that the protein have a recognition marker, which the fungal glucosidase apparently did not have. In the terminology of the mucopolysaccharidoses, the fungal enzyme was not a corrective factor for Pompe's disease.

Thus, in the purification of enzymes for potential therapy of mucopolysaccharidoses or of other lysosomal disorders, it behooves us to monitor not only catalytic activity but also the ability to enter cells where storage occurs. This is readily done when connective tissue cells are affected, since cultured skin fibroblasts provide a simple model system for correction. New techniques must be developed, however, for studying uptake by other cell types, such as hepatocytes and neurons.



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The enzymatic defects in most of the mucopolysaccharidoses have been elucidated. In each case, the deficient enzyme is a glycosidase or sulfatase needed for the degradation of dermatan sulfate or heparan sulfate: α-L-iduronidase (deficient in the Hurler and Scheie syndromes), as well as sulfoiduronate sulfatase (Hunter syndrome), β-glucuronidase (β-glucuronidase deficiency disease), heparan sulfate N-sulfatase (Sanfilippo syndrome, A subtype), and N-acetyl-α-glucosaminidase (Sanfilippo syndrome, B subtype). Each of these enzymes may be supplied exogenously to the deficient fibroblasts in culture. Uptake of the enzyme results in restoration of the catabolic pathway. This is known as "correction," and the lysosomal enzyme functions as a corrective factor for the deficient cells.

The requirement of enzyme uptake for correction has provided the key to understanding I-cell disease and pseudo-Hurler polydystrophy. These two mucolipidoses are characterized by multiple lysosomal enzyme deficiencies in cultured fibroblasts, accompanied by an extracellular excess. We can postulate that packaging of hydrolytic enzymes into lysosomes of fibroblasts requires secretion of these enzymes followed by specific recognition and uptake into adjoining cells. In the two mucolipidoses, the enzymes are not taken up (and therefore do not function as corrective factors), probably because of a defect in some structural feature needed for recognition.

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5

Approaches to the Control and Prevention of Tay-Sachs Disease

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INTRODUCTION 104
BACKGROUND AND RATIONALE 104
PROGRAM STRATEGY AND DESIGN 106
METHODS: DESCRIPTION AND ASSESSMENT 113
RESULTS AND ANALYSES 118
Epidemiologic data 118
Testing results 120
In-program evaluations of the testing method 121
Overall program results 124
INFORMING SCREENEES 125
RELATIVES OF CARRIERS 125
PSYCHOSOCIAL CONSIDERATIONS 126
GENETIC CONSIDERATIONS 128
ECONOMIC AND SOCIAL CONSIDERATIONS 129
Conclusions and Implications 131
ACKNOWLEDGEMENTS 132
REFERENCES 133

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5

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INTRODUCTION 104
BACKGROUND AND RATIONALE 104
PROGRAM STRATEGY AND DESIGN 106
METHODS: DESCRIPTION AND ASSESSMENT 113
RESULTS AND ANALYSES 118
Epidemiologic data 118
Testing results 120
In-program evaluations of the testing method 121
Overall program results 124
INFORMING SCREENEES 125
RELATIVES OF CARRIERS 125
PSYCHOSOCIAL CONSIDERATIONS 126
GENETIC CONSIDERATIONS 128
ECONOMIC AND SOCIAL CONSIDERATIONS 129
Conclusions and Implications 131
ACKNOWLEDGEMENTS 132
REFERENCES 133

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INTRODUCTION

In May 1971, the first prospective Tay-Sachs disease (TSD) prevention program was initiated in the Jewish communities of Baltimore, Maryland and suburban Washington, D.C. In the first year, 12 community-based carrier-screening sessions were conducted at synagogues, schools, and community centers in these areas. Over 7,000 persons volunteered to have the TSD heterozygote detection test. This was the first voluntary, adult-oriented, prospective, genetic screening program of its type. Our purpose in this report is to describe the technologic developments which made this program feasible, the details of the program design and educational format, certain studies which were conducted to evaluate the program, and the overall implications (both genetic and social) of such an effort.

BACKGROUND AND RATIONALE

Tay-Sachs disease is the prototype disorder of a group of diseases classified as the sphingolipidosis. This condition, first described in the late 19th century, is a progressive neurodegenerative disorder uniformly fatal at 3 to 5 years of age. Reports show that it is inherited as an autosomal recessive condition and that it has a marked predilection for Jewish infants of central and eastern European ancestry (the Ashkenazi Jews) (Myrianthopoulos, 1962).

Over the past century, a number of important developments have occurred that have increased our understanding of this disease. As early as 1887, Tay-Sachs disease was defined by histologic techniques as a neuronal lipid storage disease (Sachs, 1887), and the specific lipid (GM_2 ganglioside) which accumulates in the tissues (mainly of the central nervous system) was defined in the 1960s (Svennerholm, 1962). By ultrastructural studies, Terry and Weiss (1963) have identified intracytoplasmic lysosomal-like structures, particularly within neurons of the central and peripheral nervous systems, as the primary sites of lipid accumulation.

In the mid-1960s, Brady and his coworkers discovered the biochemical basis for two related sphingolipid disorders, Gaucher's disease and Niemann-Pick disease (Brady et al., 1965; Brady et al., 1966). In each case, tests showed the activity of a specific catabolic hydrolase to be deficient in the tissues of affected persons. Deficiency in activity of a specific degradative enzyme is believed to result in a striking increase in tissue levels of the immediate sphingolipid substrate for that enzyme. Investigators predicted from these studies that Tay-Sachs disease would also be related to a specific catabolic lysosomal hydrolase deficiency (Brady, 1966).

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In 1969, O'Brien and his coworkers discovered that activity of the

lysosomal isoenzyme, hexosaminidase A (Hex A), was totally deficient in the tissues of children with Tay-Sachs disease (Okada and O'Brien, 1969). Subsequently, these workers were able to establish that a partial deficiency of this same enzyme activity could be demonstrated in the tissues and peripheral blood of obligate heterozygotes for the Tay-Sachs gene (parents of Tay-Sachs children) (O'Brien, et al., 1970). Shortly after these discoveries, researchers successfully applied this new information to the intrauterine detection of Tay-Sachs Disease in second trimester fetuses (Schneck et al., 1970; O'Brien et al., 1971a).

In light of these advances, "prospective prevention" (before appearance of a problem in a family) of most cases of Tay-Sachs disease becomes theoretically possible. This is so because TSD meets the following three critical prerequisites:

1. *The disease occurs predominantly in a defined population.* The estimated heterozygote frequency for the gene for Tay-Sachs disease is tenfold greater in Jews of central and eastern European ancestry than in Sephardic Jews or non-Jewish populations (1 in 30 versus 1 in 300). Ashkenazi Jewish couples, therefore, have a 100 times greater risk for Tay-Sachs disease in their offspring than do the offspring born to non-Jewish couples (Myrianthopoulos, 1966) (1 in 900 Jewish couples compared with 1 in 90,000 non-Jewish matings).
2. *Heterozygotes for Tay-Sachs disease can be identified simply, accurately, and inexpensively.* A small serum sample can be quantitatively analyzed for activity of hexosaminidase A. By using the synthetic fluorogenic substrate 4-methyl umbelliferyl β-D glucosaminide (4 MUGA), hexosaminidase A activity can be quantitated because of its selective lability to heat exposure (Robinson and Stirling, 1968). Carrier detection is highly accurate by this method, since carriers have a significant reduction in Hex A activity as determined in their serum, leukocytes, and other somatic tissues (O'Brien et al., 1971b; Suzuki et al., 1971). With the development of automated techniques for the determination of serum Hex A levels (to be described subsequently), high-volume screening can readily be achieved. In this fashion, the cost per test is relatively small, making mass screening economically feasible. In addition, the accuracy of such determinations is extremely high as we will demonstrate.
3. *Tay-Sachs disease can be detected in utero early in pregnancy.* Transabdominal amniocentesis in the early second trimester provides a source of fetal somatic cells. Supernatant amniotic fluid, uncultured cells, and cultivated amniotic fluid cells can be used to detect hexosaminidase A levels and thereby to diagnose Tay-Sachs disease or its absence in the second trimester fetus (Okada et al., 1971a).

Since the above prerequisites for prospective prevention have been met in Tay-Sachs disease, it becomes the first autosomal recessive condition for which such a program is possible without imposing genetic limitations on the person's freedom of mate-selection or on the couple's freedom of choice to reproduce. Screening of child-bearing age persons in the at-risk population (Ashkenazi Jews) should permit the identification of those relatively rare couples in whom both members are heterozygous for the Tay-Sachs gene (1 in 900 Jewish couples) and who, therefore, are at-risk for the disease in their offspring. Couples determined to be at-risk could be so identified prior to the birth of an affected child by such screening. With appropriate counselling, it should be feasible then to monitor all pregnancies in these couples and, in so doing, to enable them to reproduce without fear of having children with Tay-Sachs disease (assuming such couples would elect to terminate pregnancies in which a fetus with Tay-Sachs disease was identified). In this way, the incidence of Tay-Sachs disease could be strikingly reduced and at the same time a mechanism for the selective birth of only unaffected children could be available to those couples determined to be at-risk for the condition in their offspring.

With these considerations in mind, a program design was formulated for the voluntary screening of the high-risk populations of Baltimore and Washington. It was apparent from the outset that, if screening were to be provided on a voluntary basis, there would be a critical need for an effective educational program. Alternatives such as a mandated or legislated program were considered ethically unacceptable (Kaback and Zeiger, 1972b). Accordingly, the detailed program which follows was devised.

PROGRAM STRATEGY AND DESIGN

Perhaps the most critical point in formulating a voluntary mass genetic screening program is to consider a careful definition of the program goals. If mass screening of a target community is to be achieved on a voluntary basis, it is essential to devise a strategic plan for mass community education. If education is to elicit high compliance, the timing of this educational activity might best be carried out immediately prior to or at the time the testing procedure is available. Mass public announcements about such a program well in advance of being able to provide the laboratory services involved might serve only to generate concern and possible anxiety in the community. The educational program used was carefully developed with this concept in mind and its delivery withheld until just before the onset of community screening.

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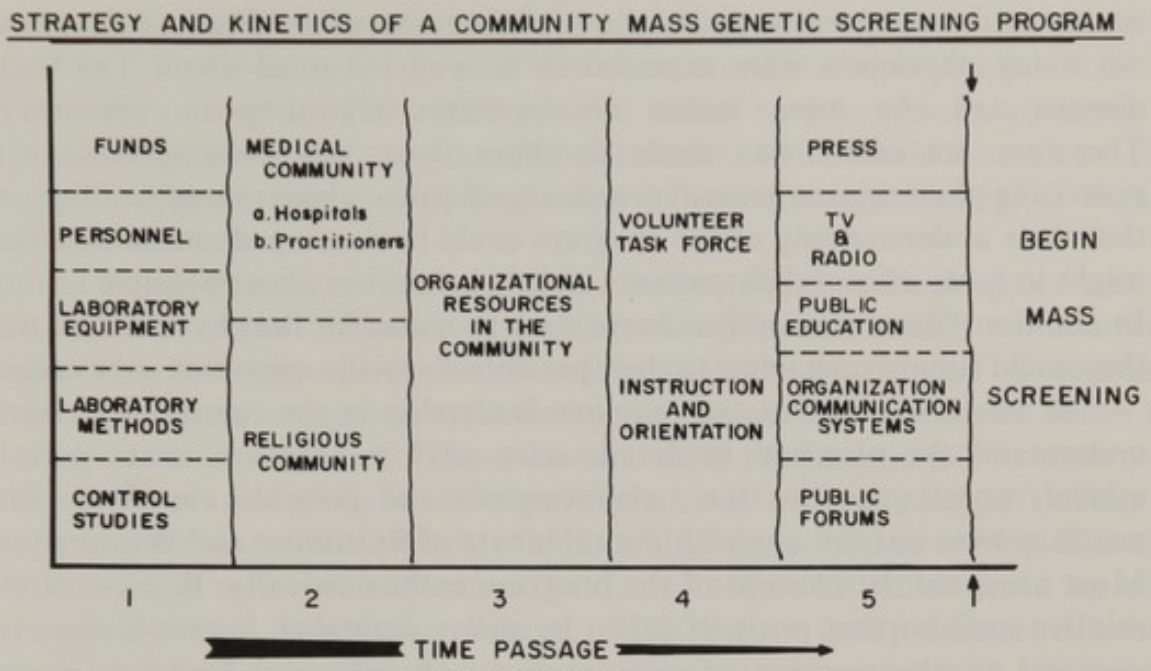
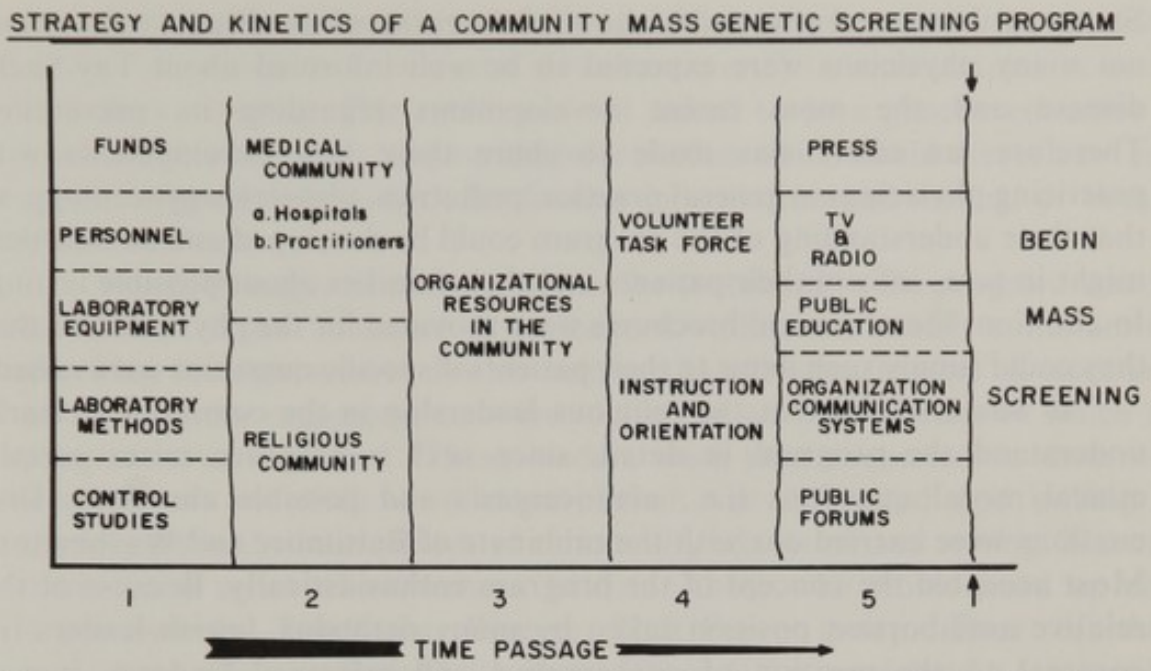


Fig. 1. Strategy and timing of the prototype Baltimore-Washington Tay-Sachs Screening Program. Phases 1 through 4 occupied a period of 14 months (before public education and screening began). Further discussion in text.

unknown disease. Once informed, they could immediately get tested if they so chose. The overall strategy and timing of the Baltimore–Washington program is depicted in Figure 1. We should point out that phases 1 through 4 were accomplished over a period of approximately 14 months; that is, 14 months of laboratory preparation, medical and religious leader education, and organizational development occurred prior to the public education phases of this program.

It was essential to develop a testing method which could process screenees in high volume. Accordingly, the initial months were spent evaluating and modifying the carrier-detection method and developing an assay system which could be delivered in high volume, with minimal expense, and with a high degree of accuracy. These efforts are described in the methods section.

For a community to become informed and responsive to a relatively "new" health issue (genetic disease and its prevention), it seemed necessary that community leadership first be informed concerning the program involved. Because of the delicate nature of this program, it was apparent that the medical practitioners and the religious leadership of the community should be addressed before education of the public was attempted. Through mailed communications and direct interactions with medical and religious groups, a detailed presentation of the program (including its goals, mechanisms, and "philosophy") was carried out. Because of the relative paucity of medical genetics curricula in most medical schools in the United



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States until very recently, and because of the rarity of the disease in question, not many physicians were expected to be well informed about Tay-Sachs disease and the most recent developments regarding its prevention. Therefore, an effort was made to share these new developments with practicing physicians in general practice, pediatrics, obstetrics-gynecology, so that their understanding of the program could be developed and so that they might in turn, inform their patients and their families about possible testing. In addition, literature and brochures were provided for the physicians so that they could supply such items to their patients if specific questions were raised.

It was essential that the religious leadership in the community clearly understand the program in detail, since such a program raises certain ethical/moral questions (i.e., anniocentesis and possible abortion). Discussions were carried out with the rabbinate of Baltimore and Washington. Most accepted the concept of the program enthusiastically. Because of the relative antiabortion position taken by many orthodox Jewish leaders (in contrast to the position of conservative and reformed leaders), it was essential that the concept of this program be clearly conveyed to these men. The program was presented primarily as a blood-testing program which could provide information to young married couples. Instructors emphasized that such information could be helpful to some families who otherwise might not be willing to have children because of fear of this disease. Moreover, physicians explained that for couples found to be at-risk, other alternatives might also exist such as adoption or artificial insemination. This was of particular significance with the orthodox rabbinate. Moreover, they learned that if a person, because of personal or religious reasons, would not consider the "positive" alternative of amniocentesis and possible abortion (if found to be at-risk), testing could be provided before marriage (if mate selection were desired on the basis of the genetic constitution of the person). With the exception of a few Hasidic rabbis, most of the orthodox leaders, according to their interpretation of the Talmud, felt that intrauterine detection, and even the possible abortion of a fetus doomed to die with Tay-Sachs disease, could be deemed acceptable.

The rabbinate participation in the program was important for several other reasons as well. First, because of the community "out-reach" nature of the program, testings were planned at community facilities. Since synagogues were to be used as a major focus for community screenings, the rabbis would have to consent to the use of their facilities for this purpose. Second, the rabbis provided the link to a most essential group, the sisterhoods. These ladies made up a substantial portion of the voluntary work force of the program. A last and most important reason for rabbinical involvement was their critical role in maintaining the screening effort. After multiple mass screenings in the community have been completed, the ongoing nature of the program was best sustained by simply testing all newly married Jewish cou-

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The next major resource group to be mobilized in the program was the leadership of the volunteer organizations within the Jewish communities. Working through various Jewish community agencies, leaders of essentially all community organizations were invited to an open forum at which the program was discussed. These leaders were then requested to determine whether or not their own organization was interested and willing to participate in the later phases of community education and testing. The next step was a series of workshops held for those who would head up each organization's Tay-Sachs Disease Task-Force group. Four of these workshops were conducted with about 500 or 600 women (and a few men) in attendance. Educational material was distributed and a careful and detailed seminar-style discussion was conducted which conveyed the simple facts of what Tay-Sachs disease is, why it had a predilection for Jewish persons, what the testing program was and what it could achieve, and what the goals and philosophical implications of the program were. In essence, these workshops were directed at developing a team of mini-experts from the community who, using their knowledge and expertise of the community and its organization, could convey an accurate and informative message to the target populations in their areas.

The public education program began only after these efforts had been completed. This took the form of a saturation effort, using multiple educational mechanisms directed at reaching all persons in the community. The systems used are shown in Figure 2. The media played an important role particularly in reaching persons who did not belong to organizations or congregations which were not represented by task force educational groups.

The program design provided a mechanism by which the community was involved in the planning, organization, and delivery of the program from its very inception. A substantial proportion of the funds to support the program were developed in the private community, as well. This also gave the community involved a sense of program identity. The authors believe that these concepts in organization, education, and so forth will be important in future genetic screening programs which relate to other specific ethnic, national, or religious groups (i.e., sickle cell, thalassemia, or cystic fibrosis).

By enabling all interested organizations and groups in the community to participate, the total community identification with the program was reinforced. Testings were planned sequentially and groups of organizational task forces were brought together to work on each independent testing. This

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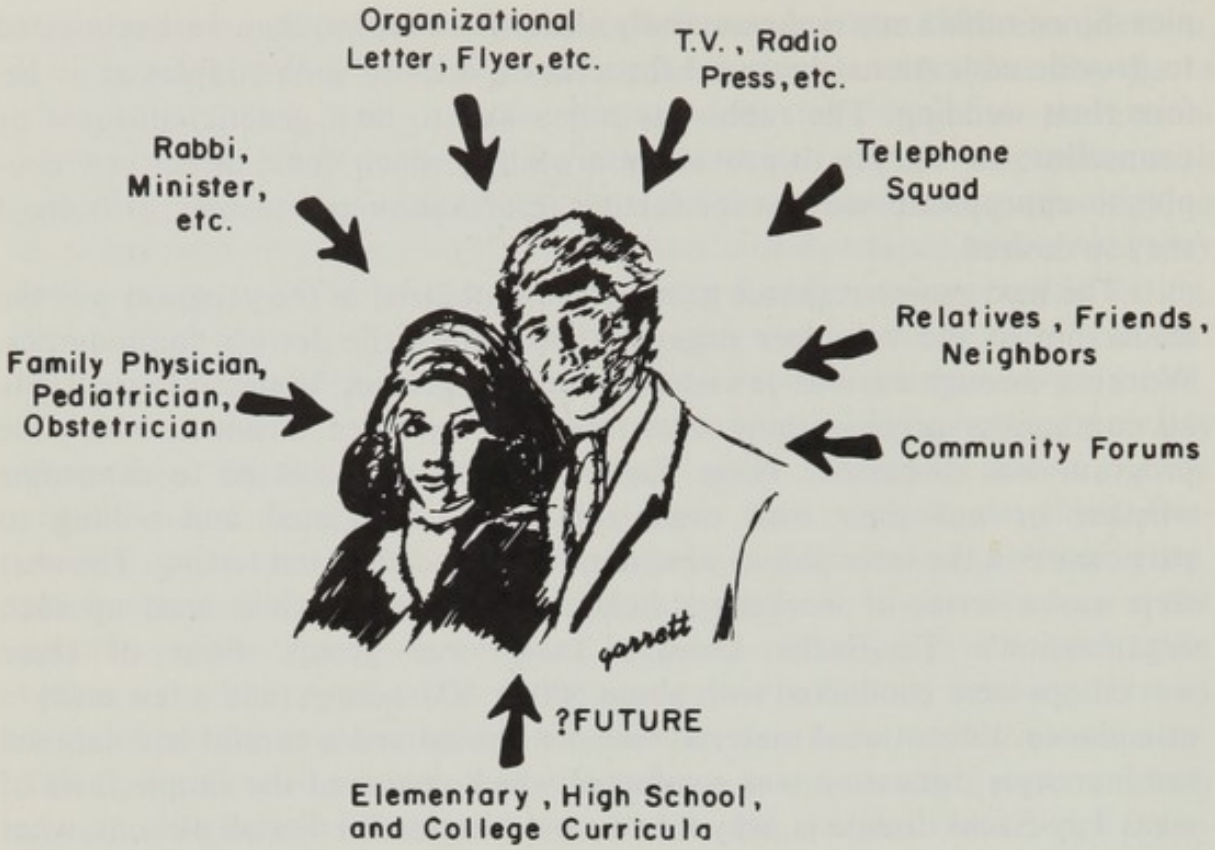

Fig. 2. Multiple means used in community education. A "saturation" approach.

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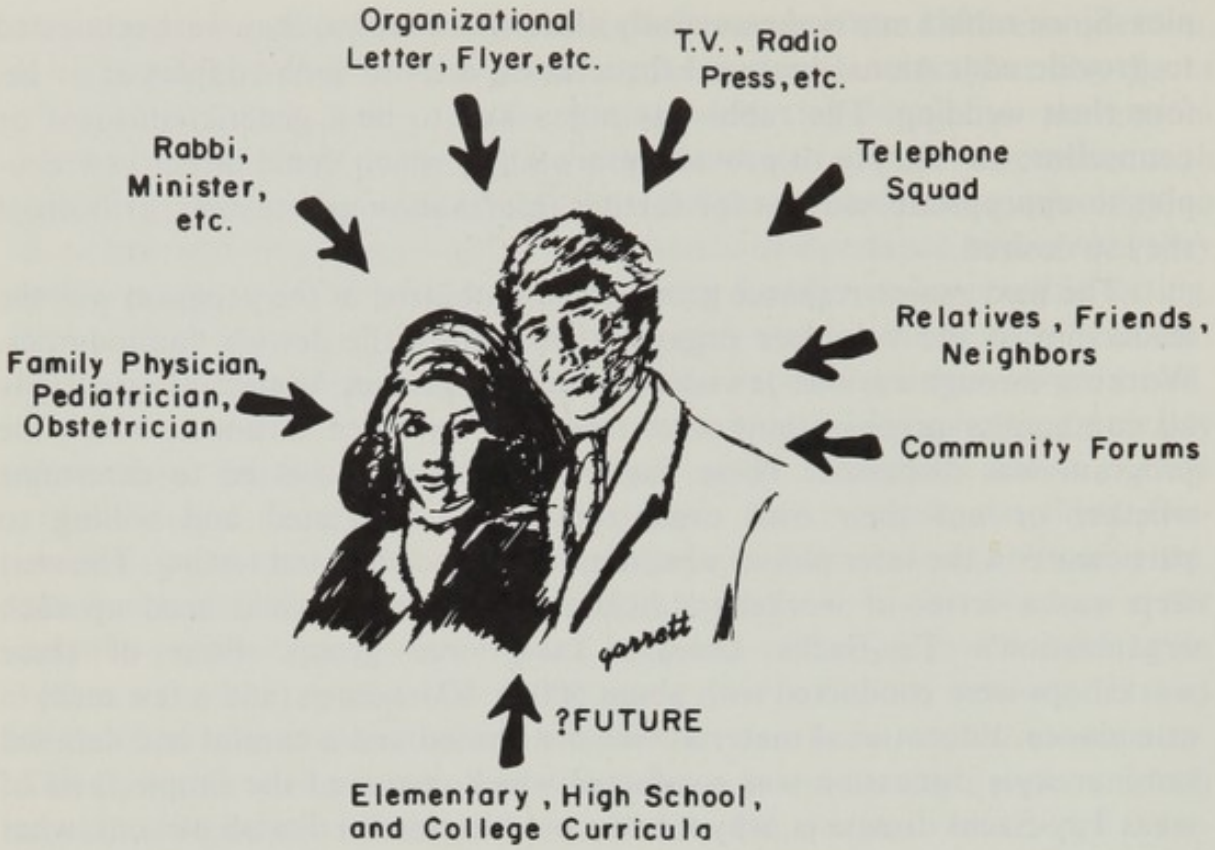
A group of organizations would be designated to participate in a particular testing effort (see Fig. 3). These groups would meet on two occasions with the program director. The first meeting, about 4 to 6 weeks prior to the actual testing, would be to reinforce the educational program and to discuss possible strategies for education of their own organizations and people within their immediate community. The second meeting would occur in the week prior to the testing date itself. At this session, a detailed discussion was carried out regarding the needs, types of jobs, and responsibilities of volunteers who would work at the testing sessions. All testings were held either on weekday evenings (i.e., 7:00 to 10:30 PM) or on Sunday afternoons (2:00 to 5:00 PM) to facilitate maximum response.

Approximately 70 to 80 volunteers served at each testing session. Between 10 and 15 physicians and technicians manned the testing stations. Volunteers did all recording, tube handling, and labeling. It is a testimony to the motivation and enthusiasm of this volunteer group that with over 7,000



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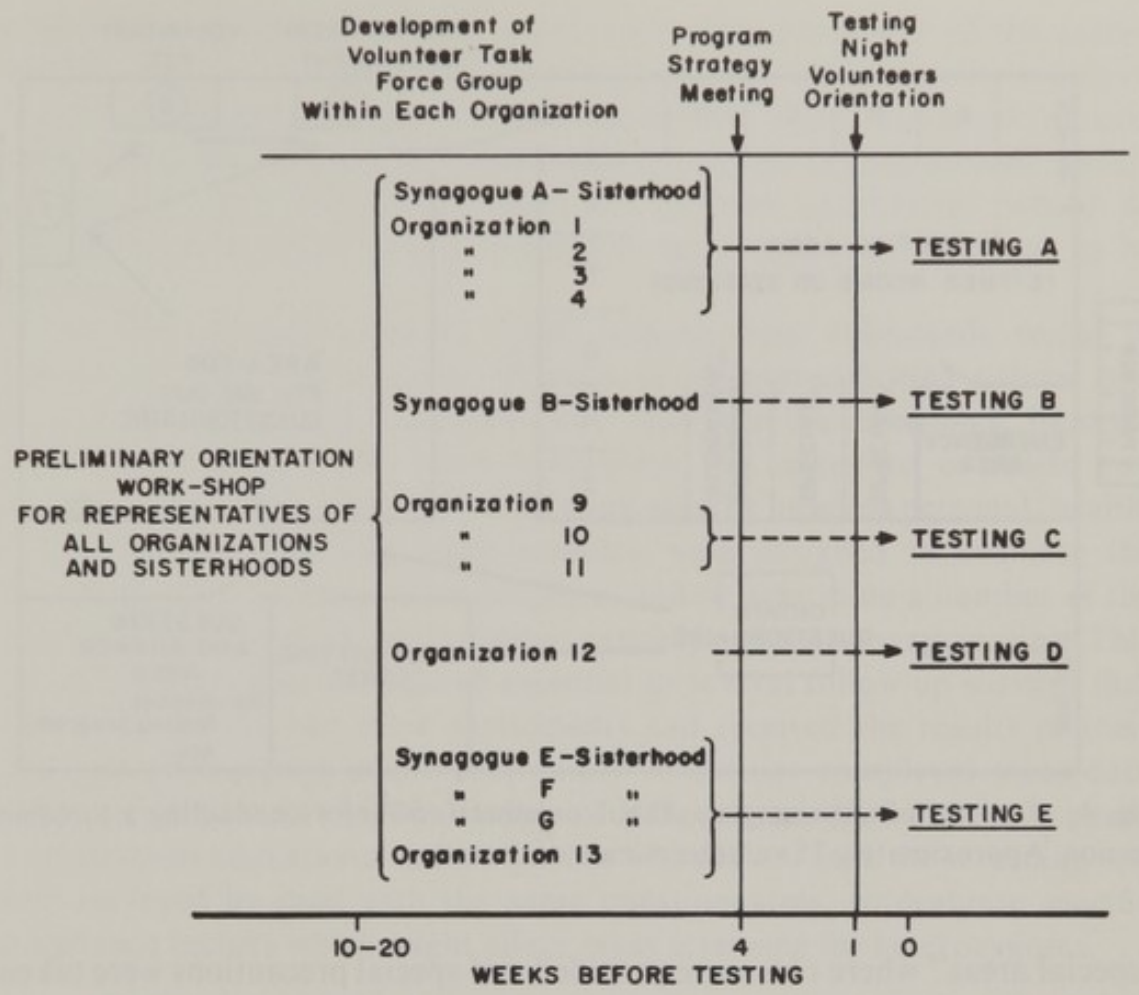

Fig. 3. Sequence and means for multiorganizational participation in the community screening effort. Different groups are involved for different target testings.

samples collected in this fashion, only four tubes were misnumbered or mishandled. As many as 1,800 persons were processed at one testing session (5 hr) without significant delays or confusion.

The layout of a community facility for such a screening session is shown in Figure 4. As previously mentioned, all interaction with the public was primarily handled by task-force volunteers. Participants were requested to fill out questionnaires, or they were directed to counselling areas if they had questions; and generally they moved through the testing session in a highly efficient fashion. After completing questionnaires, the participants were registered and then if they wished, they paid a voluntary fee of $5.00 per person for the test. No billing was made for this test and the fee was requested on a completely nonobligatory, voluntary basis. (Over 93 percent of the people paid for the test.)

Testing area assignments were again given by volunteers after ascertaining if any medical problems or undue anxiety was present in the person to be tested. If either was evident, participants were directed to the

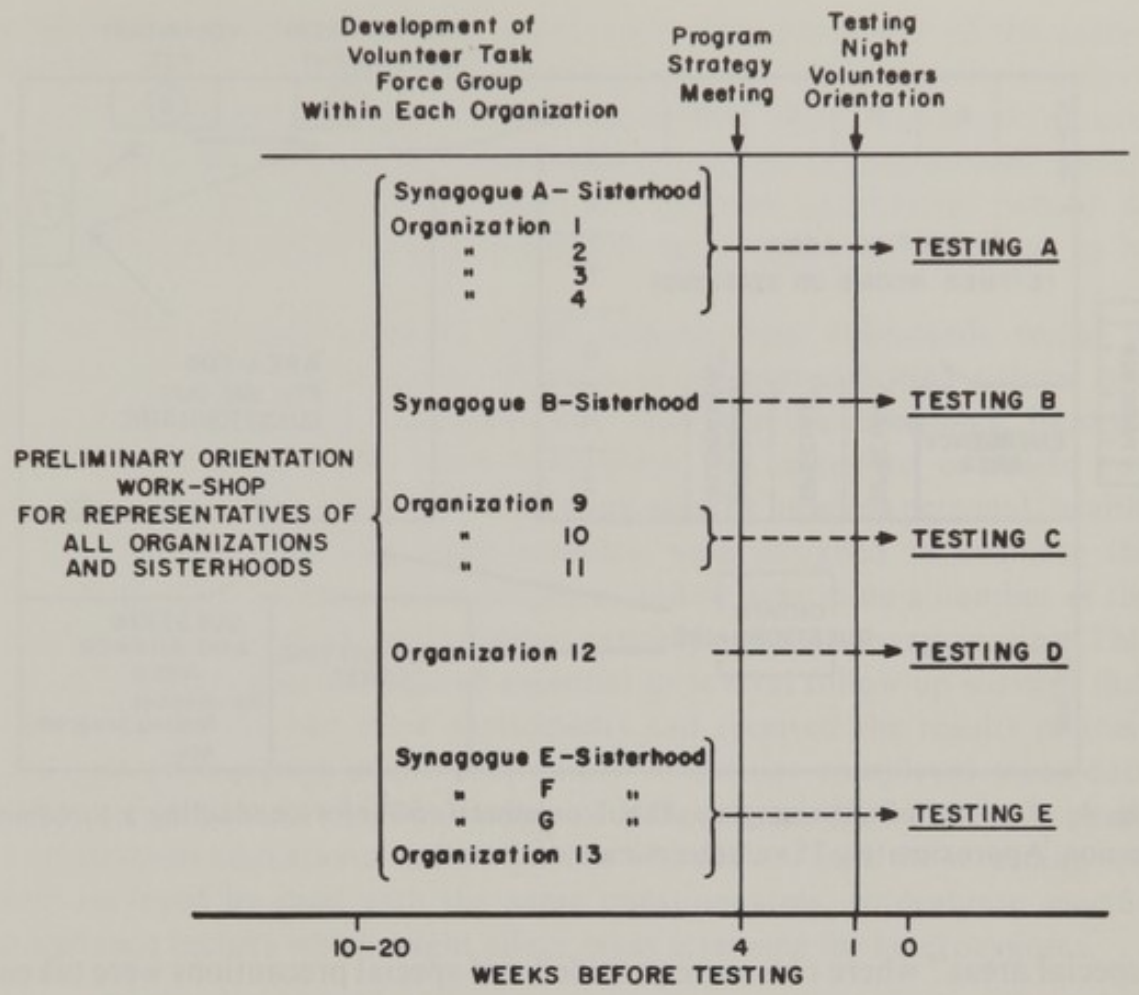

Fig. 3. Sequence and means for multiorganizational participation in the community screening effort. Different groups are involved for different target testings.

samples collected in this fashion, only four tubes were misnumbered or mishandled. As many as 1,800 persons were processed at one testing session (5 hr) without significant delays or confusion.

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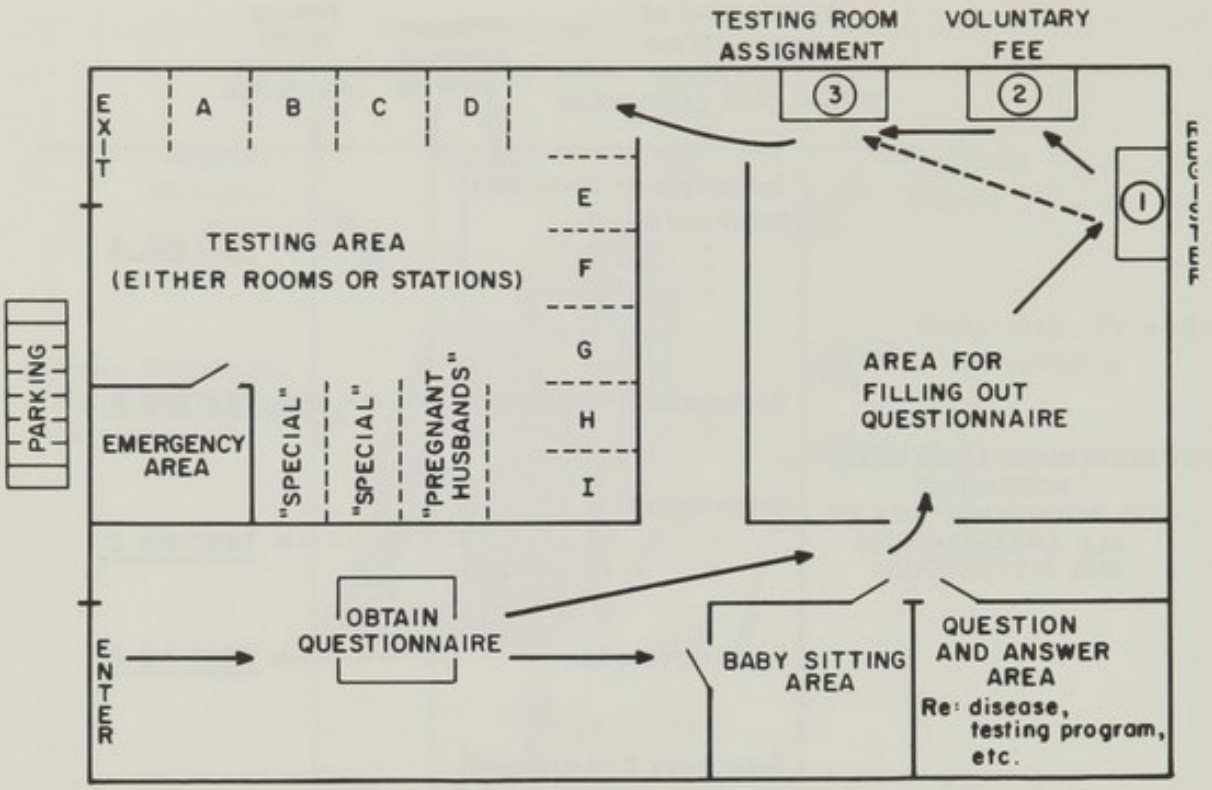


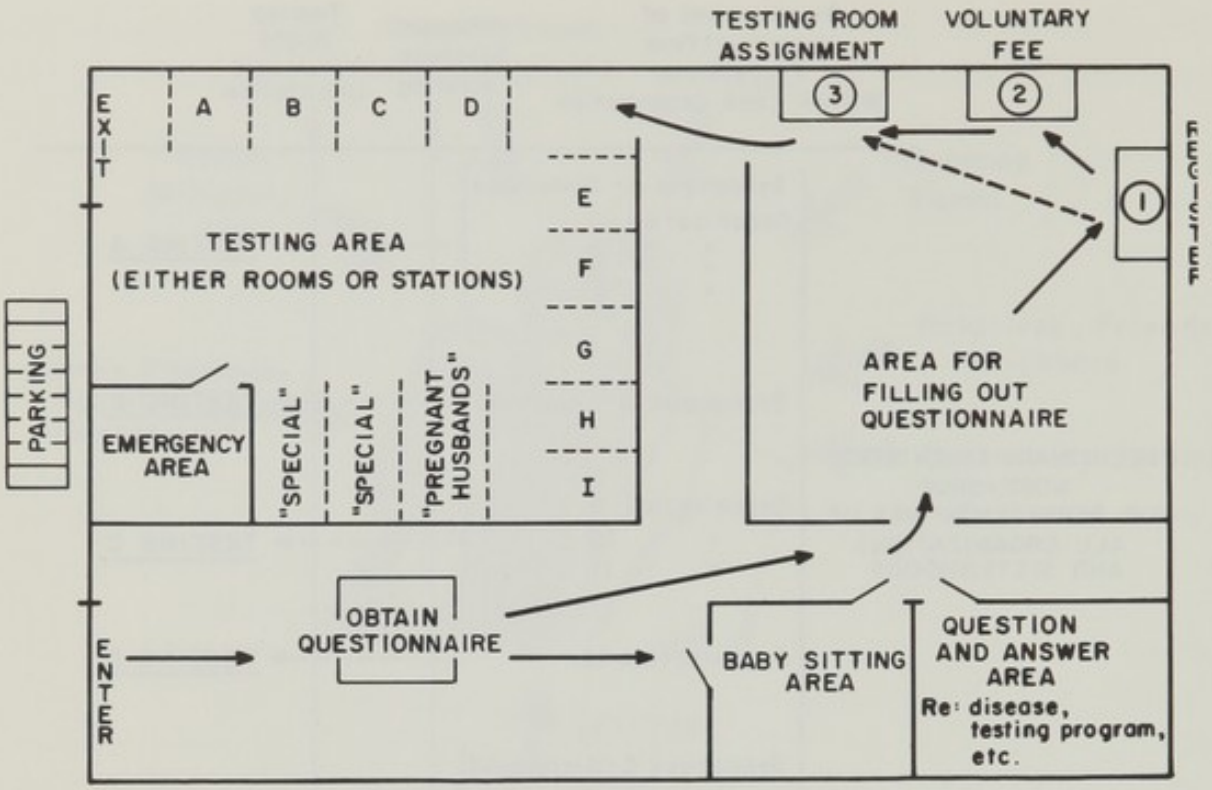
Fig. 4. Lay-out of a synagogue, school, or other facility for conducting a screening session. Approximately 75 volunteers man each testing.

"special areas" where cots were provided and special precautions were taken. (Only three subjects—all men—fainted during these sessions.)

Each man was questioned several times before testing to determine whether his wife was pregnant at that time. If the wife was more than 4.5 months pregnant, neither she nor her husband was tested. If the woman was fewer than 4.5 months pregnant, only her husband was tested. He was deemed a "pregnant husband" and sent to one specific area in the testing facility. His blood which was kept separate from that of others at the testing site was assayed immediately, so that within 2 to 3 days of the testing every "pregnant husband" would have results of his test. If such test results were either positive or inconclusive as to carrier status, the husband and his pregnant wife were immediately contacted by phone and requested to come to the hospital facility for leukocyte carrier-detection testing.

In this fashion, as many as 2,000 persons could be managed at any given 4- to 5-hr screening session. The number of participants to be screened at a testing could be controlled (somewhat) either by a limited educational program directed at specific organizations or community subpopulations (i.e., one apartment complex), or by requesting a "called-in" appointment during the weeks prior to the testing date. This was used on several occasions to limit the total number of persons coming to that testing.

All *single persons* were counselled prior to proceeding through the testing facility. The possibilities for potential psychological hazards with single-person carrier identification were discussed with each person. If the



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person still wanted to be tested after a complete discussion of the carrier detection test and its potential implications (e.g., possibly creating problems in social life, dating), this was obviously permitted. Approximately 90 percent of the singles who voluntarily came to the centers for testing decided *against* testing after such discussion. Since a "positive alternative" would be available to any couple—most decided to wait until after marriage to be tested.

A total of approximately 7,000 persons were voluntarily tested in tthe first year of this program. Because of its unprecedented nature, several psychosocial evaluation studies were built into the screenings. Immediately before being tested, each participant was asked to complete two questionnaires for purposes of establishing specific baseline personal, health, and attitudinal data. The questionnaires were designed to evaluate the educational effectiveness of the program, and to determine a number of the personal, social, ethical, and religious attitudes of those participating. This information was also considered essential to several follow-up surveys that were to be carried out after participants had received the results of their tests. All participants in the community screenings completed these data forms. In addition, a random sample of 500 persons who had been exposed to an identical educational campaign, but who did *not* take the screening test were surveyed by mail with the same questionnaires, to evaluate specific compliance factors which might affect mass screening for heterozygotes.

METHODS: DESCRIPTION AND ASSESSMENT

Before launching the organizational and educational programs described in the previous section, substantial studies were initiated in the laboratory to evaluate the heterozygote screening method. Several modifications of the previously described O'Brien heterozygote detection method were made. Kaback (1972) has described the manual serum method with these modifications. The major changes introduced in both the manual and the semiautomated assay of hexosaminidase A relate to the buffer (P-C) concentration used (decreased from 50 mM phosphate and 30 mM citrate to 20 mM phosphate and 12 mM citrate at pH 4.4), the temperature of heat inactivation (52 C instead of 50 C), and the substrate concentration in the final assay mixture (increased from 0.67 mM to 2.0 mM 4-MUGA). These minor modifications of the heat-inactivation method make the detection of heterozygotes for the Tay-Sachs gene highly accurate.

A semiautomated method was also developed to perform this determination. This method is a dual-sampler, dual-fluorometer, Technicon autoanalyzer system, which is a highly reliable and reproducible method for screening up to 300 persons per day (Kaback, M. M., Reynolds, L. W., and Lash, E., manuscript in preparation). After 10-fold serum dilution in P-C

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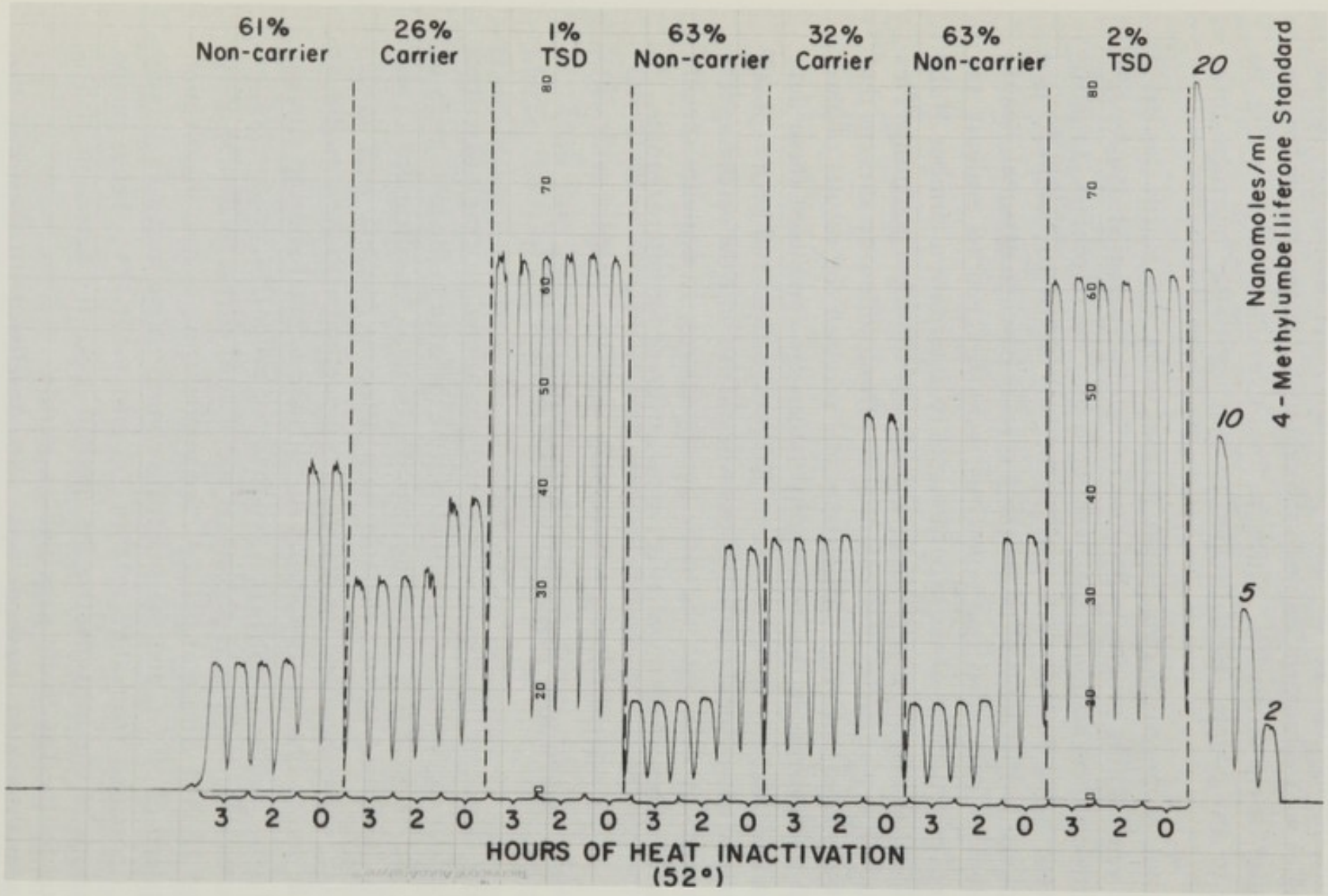
61%
Non-carrier
26%
Carrier
1%
TSD
63%
Non-carrier
32%
Carrier
63%
Non-carrier
2%
TSD
20
10
5
2
Nanomoles/ml
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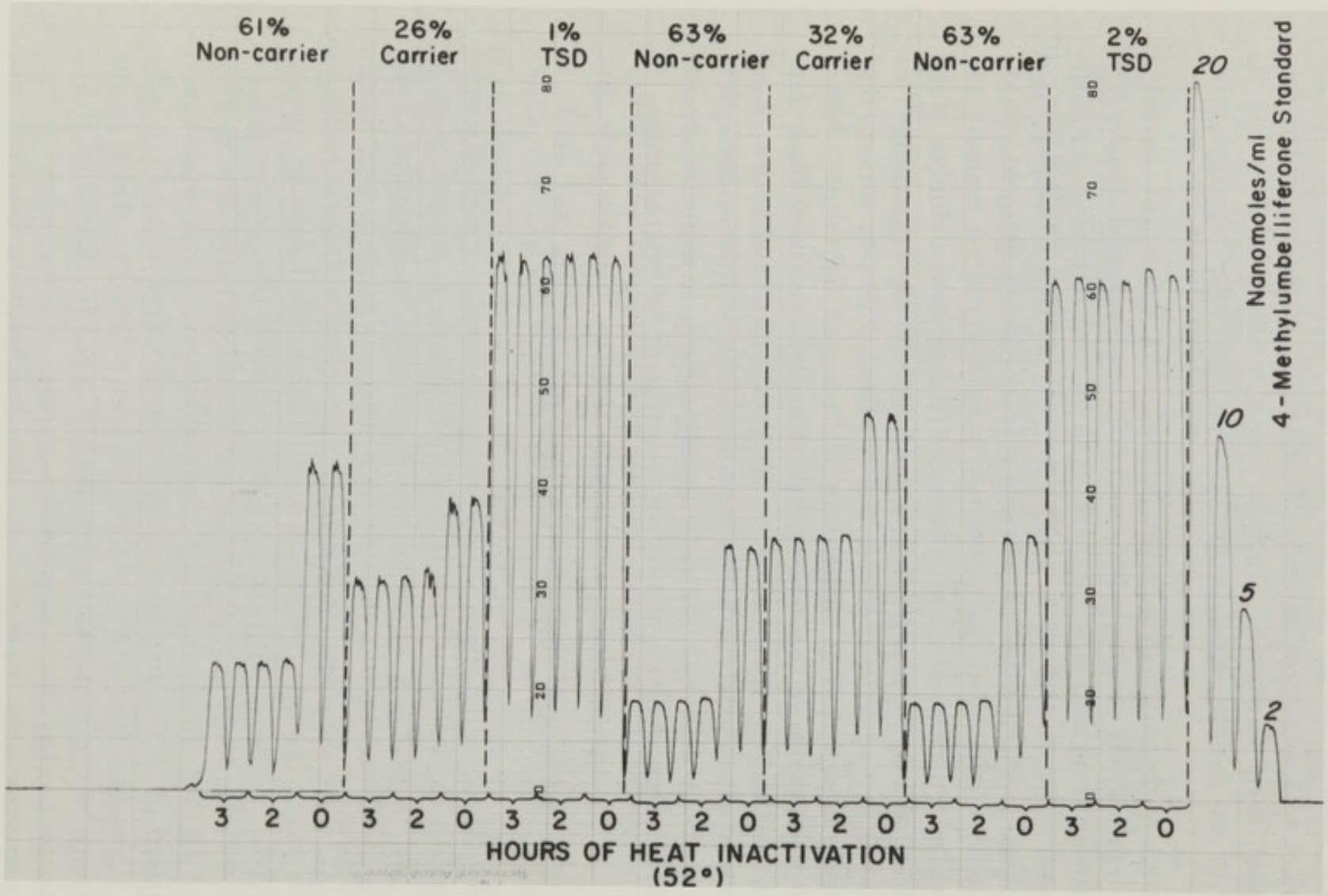
3 2 0 3 2 0 3 2 0 3 2 0 3 2 0 3 2 0 3 2 0
HOURS OF HEAT INACTIVATION
(52°)


61%
Non-carrier
26%
Carrier
1%
TSD
63%
Non-carrier
32%
Carrier
63%
Non-carrier
2%
TSD
20
10
5
2
Nanomoles/ml
4-Methylumbelliferone Standard
3 2 0 3 2 0 3 2 0 3 2 0 3 2 0 3 2 0 3 2 0
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In addition to the need for a simple method for carrier assessment, it was essential to critically evaluate the accuracy of the method. Since the only control for heterozygote determination is a parent of a Tay-Sachs child, a large number of families with Tay–Sachs children were contacted and informed of the plan. Most of them agreed to provide serum samples, and moreover, a number of other relatives in these families were similarly tested. Serum samples were then subjected to the modified Hex A determination in a coded fashion. Based on Mendelian probabilities, an expected heterozygote frequency could be calculated for relatives of varying degrees of relationships to affected patients. The results of this evaluation are shown in Table 1. As indicated, a near-perfect correlation exists between the expected and observed carrier frequencies. This study suggests that the serum method neither overidentifies carriers (false-positives) nor significantly misses them (false-negatives).

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Table 1. Results of Serum Testing in Relatives of TSD Patients

Relationship of the Individual Tested to the TSD Child	Carrier Frequency Expected	No. Tested	Noncarriers	Carriers	Carrier Frequency Observed
Parent	1.00	68	0	68	1.00
Sibling	0.67	23	7	16	0.69
Grandparent, aunt, uncle	0.50	30	15	15	0.50
Niece, nephew	0.33	28	19	9	0.32
First cousin, grandaunt, granduncle	0.25	39	29	10	0.26
Second cousin	0.125	53	44	9	0.169
Third cousin	0.063	15	14	1	0.066

Fig. 5. A typical recording of the automated hexosaminidase assay. Heat inactivation is conducted before loading on the samples. No wash is required between samples; duplicate assays are highly reproducible.

Table 2. Serum Hexosaminidase Activity

Subjects Tested	No. Tested	Total Activity* Mean	SD	Range	% Hex A (52 C) Mean	SD	Range
Infants with TSD	13	588.4	252.0	227.4– 926.4	1.7	1.1	0 – 5.6
Obligate heterozygotes	35	655.0	221.4	279.8–1109.7	36.1	5.1	27.0–44.2
"Noncarriers"	100	842.3	166.7	440.4–1312.4	56.2	4.1	50.0–71.3

*nmoles 4-methylumbelliferone produced per milliliter of serum per hour.

depicted for the three groups: infants with TSD, obligate heterozygotes, and probable noncarriers. The serum levels of *total* hexosaminidase activity do not distinguish the heterozygote from the noncarrier. However, a clear distinction is evident when hexosaminidase A (by heat inactivation at 52°C) is compared.

From these studies a theoretical distribution was derived from the serum Hex A levels determined in 43 obligate heterozygotes for the Tay-Sachs gene (Fig. 6). Genotype designation is made on a statistical basis relative to these data. Any "unknown" person whose serum Hex A level is 3 SD or more above the mean for obligate heterozygotes is designated a noncarrier. Statistically, one can estimate that such a person would have about a 1 in 1,000 chance of being in the heterozygous distribution. Since an Ashkenazi

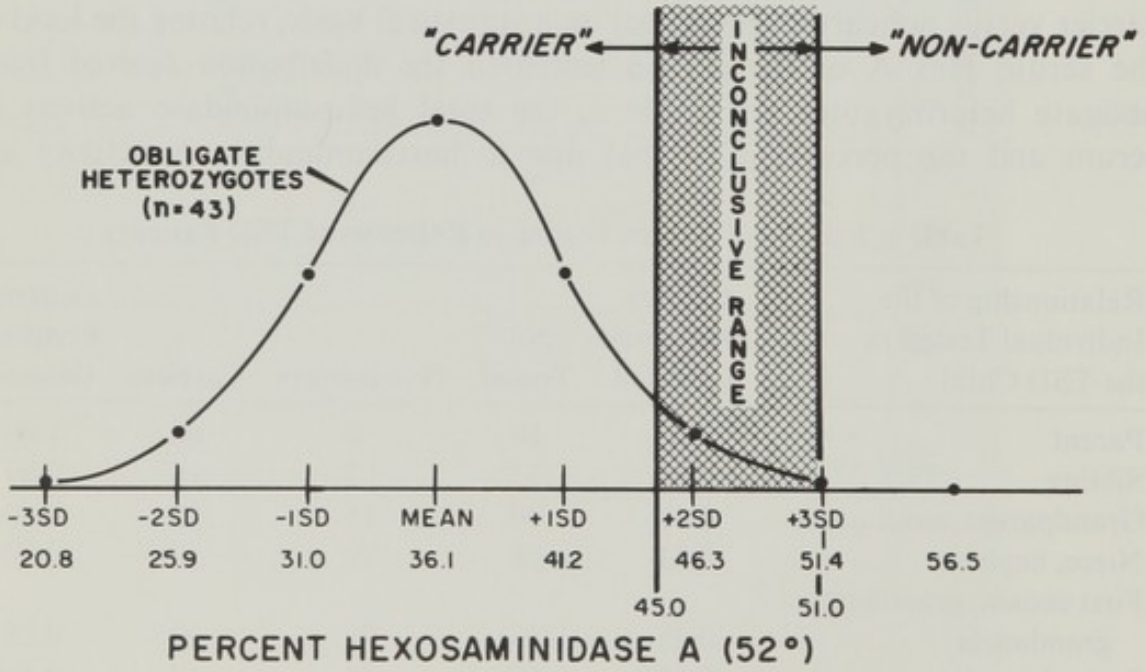

Fig. 6. Serum Hex A and carrier/non carrier designation. A statistical basis for genotype assignment is derived from normalized distribution curve of Hex A levels in serum from obligate heterozygotes. An inconclusive area is indicated which is arbitrarily defined. Participants with serum Hex A levels in this range require the follow-up leukocyte test before genotype is designated. Further description in text.

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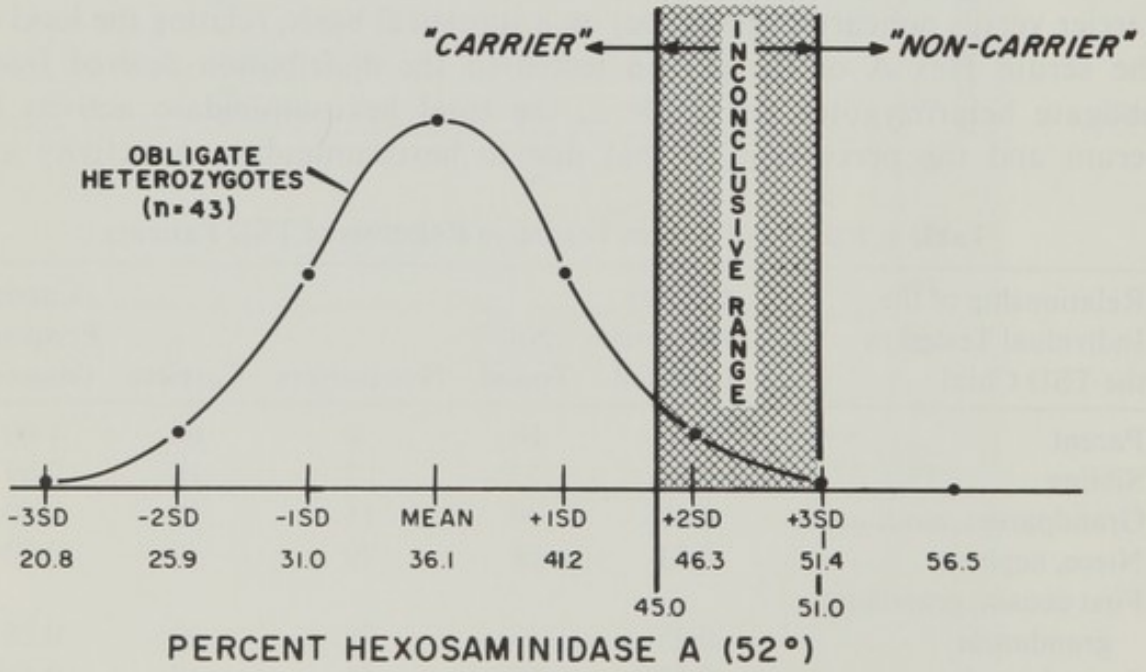

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Jewish person has approximately a 1 in 30 risk of being a carrier at the outset, then a person with a serum Hex A level 3 SD or more above the mean for known carriers, would have about a 1 in 30,000 chance of actually being a carrier for the Tay-Sachs gene. This level of accuracy is obviously acceptable.

It was known that certain conditions such as pregnancy, tissue destructive disorders, and diabetes could affect the activity of this enzyme (Woollen and Turner, 1965). We queried whether certain medications might also affect serum enzyme activities. Therefore, considerable thought was given as to how carrier designation could be made from serum assays. We decided arbitrarily that if a person's serum Hex A level fell within 1.7 SD of the mean for known carriers on three consecutive duplicate determinations from a single serum sample, then that person would be designated a carrier. However, if the person were pregnant, or had diabetes, or a tissue destructive disease (as would be apparent on the questionnaire filled out at the time of testing), such a person would not be designated a carrier until retested by leukocyte Hex A analysis (which studies show are unaffected by these conditions) (Kaback and Zeiger, 1972). Fortunately, in testing a young population like this, such problems are rare.

In pregnant women (< 4.5 months) and in certain women on oral contraceptives, the total serum hexosaminidase activity is increased and a relative decrease in percent Hex A (by heat inactivation) occurs. Thus, a high proportion of such women would falsely appear to be carriers. Leukocyte total activity and percent Hex A are not so affected, as shown in Table 3. The methods for leukocyte preparation (Skoog and Beck, 1956) and determination of Hex A have previously been described (Kaback, 1972). It is not clear what factors affect the serum hexosaminidases, but since these are apparently enzymes of lysosomal origin, it is clear that the serum is a secondary site in which to measure enzyme activity. This may explain why

Table 3. Serum and Leukocyte Hexosaminidase in Pregnant* and Nonpregnant Women

Subjects Tested	No. Tested	Serum Hex. Total	Serum Hex. % A	WBC Hex. Total	WBC Hex. % A
Obligate heterozygotes					
Nonpregnant	6	443.9– 829.9	35.6± 4.7	453.7–618.9	36.8±3.6
Pregnant†	6	733.4–1486.1	24.3± 6.5	510.9–863.8	37.4±4.3
"Controls"					
Nonpregnant	59	354.1– 926.3	57.2± 4.9	304.2–1007.2	61.8±4.0
Pregnant	43	561.2–1305.0	35.9±11.1	469.3–1687.9	66.2±6.0

*Between 6 to 24th menstrual week of pregnancy.

†Same 6 women.

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When serum Hex A levels fall between 1.7 and 3 SD above the mean for known heterozygotes, the person is arbitrarily called *inconclusive*. These persons are also subjected to a follow-up leukocyte test. In essentially all such persons, an accurate genotype designation can be made after the more definitive leukocyte study. These data are discussed further in the next section on results and analyses.

RESULTS AND ANALYSES

After completing the methodological and organizational efforts, public education and mass screening began. At the time of preparation of this report, nearly 10,000 persons in the Baltimore and Washington areas had voluntarily been tested. The results of the first 12 community testing will be discussed in detail here. This experience includes approximately 7,000 persons.

Epidemiologic Data

Fifty-six percent of those tested were women and 44 percent were men with a great predominance of married persons in this tested population (94 percent) (Table 4). Approximately 80 percent of the group were tested as couples. When the tested population is analyzed by sex and age distribution (Table 5), it is apparent that the younger age groups predominate. This is as might be expected since the education program was directed at couples of child-bearing age. The small number of single participants tested reflects the program "bias" (as previously described) against testing unmarried persons. The predominance of younger married couples perhaps reflects their interest in a genetic screening program which provides alternatives regarding future family planning. Accordingly, couples in the older range of the child-bearing

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Table 4. Epidemiologic Factors in a Voluntary Tay-Sachs Screening Program of 6,938 Subjects

Participants	%
Females	56
Males	44
Married	94
Engaged	5
Single	1

age population may well have completed having their families, and therefore would be less interested in pursuing such a genetic testing service.

In both the Baltimore and Washington communities over 94 percent of the persons tested were Jewish. More than 90 percent of those tested indicated Russian, Polish, or German ancestry. If one includes the Czechoslovakian, Hungarian, Bulgarian areas, approximately 95 percent of the Jewish persons tested would be classified as Ashkenazi Jews.

The educational level of the tested population was extraordinarily high. Approximately, 75 percent of those tested were either graduates of college or college and postgraduate programs. Less than 1 percent had not completed a high school education.

This population therefore can be characterized as predominantly married young Jewish couples with a high level of educational background. The distribution of the screenees reflects a predominance of persons under 30 years of age. The differences in age between men and women probably reflects the younger age of women at the time of marriage since this is consistent in each of the brackets indicated. In addition, the relative predominance of females tested suggests a greater willingness (and possibly interest) in women to have a test relating to family planning or a greater hesitance or perhaps fear among men regarding blood tests. Obviously, other

Table 5. Sex and Age Distribution of 6,938 Participants Screened in Voluntary Tay-Sachs Testing Program

Age (years)	Males (%)	Females (%)
<20	0.2	13.5
20–24	24.2	34.2
25–29	34.8	35.2
30–34	23.6	9.9
35–39	9.2	4.1
40+	8.0	3.1

Table 4. Epidemiologic Factors in a Voluntary Tay-Sachs Screening Program of 6,938 Subjects

Participants	%
Females	56
Males	44
Married	94
Engaged	5
Single	1

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explanations are also possible. The predominance of women is even further enhanced in that in nearly 250 situations, only the husband was tested in the couple because the woman was pregnant at the time of testing.

Testing Results

The results of the first 11 community-based testings are shown in Table 6. The overall heterozygote frequency determined in the 5,600 persons tested at these sessions was 0.042 (1 in 23.7 people tested). It is of interest to note that testings I, III, and IV were carried out in the Washington, D.C. area, whereas the remainder of the testings were held in Baltimore. Testing VIII was conducted at the Johns Hopkins Hospital for hospital staff and medical students. It is interesting to note that the carrier rate in the Washington testings and the Johns Hopkins facility is significantly lower (less frequent) than in the Baltimore Jewish community testings. Investigators believed that this difference may be significant in that it reflects the makeup of the respective communities involved. The Washington Jewish community is a highly transient, cross-sectional community. On the other hand, the Baltimore Jewish community is a very stable one in which many families have intermarried over a number of generations (Associated Jewish Charities of Baltimore, 1968). The difference in carrier rate between these communities may also be accounted for by differences in the seed population of the Baltimore community (predominantly Russian and Polish). This hypothesis is being studied further at this time.

Table 6. Community Tay-Sachs Screening Programs: Results of Testings I through XI

Test No.	Carrier/Total	Heterozygote Frequency	Carrier Rate
I	40/1228	0.0325	1 in 30.8
II	23/427	0.0538	1 in 18.6
III	14/382	0.0366	1 in 27.3
IV	23/649	0.0354	1 in 28.2
V	29/479	0.0605	1 in 16.5
VI	23/500	0.0460	1 in 21.7
VII	21/439	0.0478	1 in 20.9
VIII	2/65	0.0307	1 in 32.6
IX	14/298	0.0469	1 in 21.3
X	11/260	0.0423	1 in 23.6
XI	35/834	0.0419	1 in 23.9
Overall	235/5561	0.0422	1 in 23.7

Note: Participants with TSD in family excluded from calculation. Also persistent or unretested inconclusives are excluded.

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Table 7. Results of 11 Community-Based Tay-Sachs Screening Sessions

Results	Noncarriers	Inconclusive	Carrier	Total
Initial results	5248	280	209	5737
Inconclusives retested (216/280)	176	4	36	216
Final results	5424	4	245	5673

In Table 7 the overall testing results from these 11 community-based sessions are shown. The difference in the total number of persons tested (5,737 versus 5,561 in Table 6) reflects those persons who reported that Tay-Sachs disease had occurred somewhere in their family or whose tests were inconclusive and who were not retested; these persons were excluded from the Table 6 calculations. Also, several office-tested or mail-tested persons are excluded. Less than 5 percent of the persons initially screened had Hex A levels in the arbitrarily defined inconclusive rage. On retesting 216 of the 280 persons whose tests results were inconclusive, 176 proved to be noncarriers and 36 proved to be carriers. Test results for four persons were persistently inconclusive in both serum and leukocytes. Interestingly in three of these four subjects, similar studies were done in their parents. In each case one of the parents also had intermediate levels of serum and leukocyte hexosaminidase A. It is likely that these three persons represent heterogeneity for the Hex A locus. Further studies are being conducted to evaluate this possibility.

In-Program Evaluations of the Testing Method

Since investigators did not know at the outset of the program whether birth control medications would affect the hexosaminidase A levels as pregnancy does, a careful evaluation was carried out on all women who indicated use of birth control medication. The results of these studies are shown in Table 8. The percentage of total women using birth control pills (BCP), as evaluated in the first two testings (1,676 persons), showed that 18 percent of the noncarrier women were using birth control medication. When similar comparisons were made among women who proved to have inconclusive tests or who were carriers, a clear trend was established. The proportion of inconclusive women and an even greater percentage of women carriers were taking birth control pills. Clearly, the pill was distorting the serum test. None of the other commonly used drugs showed this effect when analyzed in a similar fashion. Such comparisons were carried out on aspirin, sleeping pills, cold preparations, thyroid drugs, tranquilizers, antihistamines, and corticosteroids. Accordingly, all women, whose serum levels were inconclusive or in the carrier range and who indicated on their questionnaire

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Table 8. Distribution of Female Phenotypes According to Use of Oral Contraceptive Medications (BCP)

Participants	Noncarriers*	Inconclusives†	Carriers†
Total women	722	154	78
Total on BCP	134	55	32
Ratio	0.18	0.35	0.41

*Data derived from all noncarriers from only testings I and II. Total screened: 1676 women (1544 noncarriers).

†Includes all inconclusives and carriers from testings I through IX. Total sample: 4550 women.

that they were taking BCP, were informed of this possible false-positive test and were retested with leukocyte assays.

On follow-up study, as indicated in Table 9, women whose serum Hex A levels were in the carrier range were retested with the leukocyte assay. In over 20 percent of these women, leukocyte levels were in the noncarrier range. Furthermore, on testing the parents of such BCP false-positive women, neither parent (by serum or leukocyte determination) was in the carrier range thereby confirming their noncarrier status.

In light of the previously described reservations concerning the serum carrier-detection method, two additional studies were carried out to evaluate critically this system for heterozygote detection. After the first two screenings had been completed, participants who had been identified as carriers by the serum assay were contacted and tested further. Leukocyte assays or testing of both parents of these presumed heterozygotes (by the serum assay) were done. If the leukocyte Hex A level in at least one of the parents of each person was in the carrier range, this confirmed their carrier designation. As illustrated in Table 10, 96 serum-identified carriers were so reevaluated. All of the 49 male carriers, identified by the initial serum-screening assay, proved to be carriers by either or both these follow-up methods. In 10 of 47 women so evaluated, either their leukocyte Hex A level was in the noncarrier range, or neither of their parents showed carrier level serum and leukocyte Hex A

Table 9. Reevaluation of Serum Identified Female Carriers with Regard to Use of Oral Contraceptive Medications

Subjects Tested	+ BCP	− BCP
Serum-defined female carriers	48	57
Reevaluated	47	43
Proved carrier by further study*	37	43
Serum false-positives	10	0

*Leukocyte Hex A level and/or parental testing.

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Table 10. Reevaluation of Serum-Identified TSD Heterozygotes

		Leukocyte Hex A		Parental Studies		
Sex	No. Evaluated	Carrier	Non-carrier	One Carrier	Neither Carrier	False Positives
Males	49	40/40	0/40	29/29	0/29	0/49
Females	47	32/41	9/41	23/30	7/30	10/47*

Note: Reevaluation of heterozygotes assessed by WBC Hex A levels, by parental testing, or by both.

*All 10 of these women were taking oral contraceptive medication. One was not leukocyte tested, but both parents were noncarriers.

levels. All 10 of these "false-positives" were women on birth control medication. Of course, the possibility of false paternity or other explanations for apparent false-positives should also be considered. However, in each of these women the leukocyte levels were in the noncarrier range, thereby supporting the false-positive serum tests.

A second level of evaluation of the serum method was carried out by family studies among serum-identified carriers. Here again, genetic analysis provides great strength in supporting the accuracy of these methods for heterozygote detection. When parents of program-identified carriers were tested, 51 percent of the 121 tested proved to be carriers. Interestingly, of 60 elderly couples that were tested, 2 had double heterozygotes for the Tay-Sachs gene (without ever having a Tay-Sachs child). This is as expected. In addition, on testing the siblings of program-discovered serum carriers, an expected carrier frequency of 50 percent was approximated. These data are shown in Table 11.

These studies indicate that with appropriate precautions (re: pregnancy or BCP) and with the methods used for serum assay, genotype assignment, and follow-up testing, a highly accurate determination can be made in almost every person tested. These data suggest that false-negative tests would be extremely rare (1 in 30,000 "noncarriers") and false-positives would also be highly unlikely if leukocyte retesting were done where indicated. By using this approach, no *proved* false-positive serum carrier (leukocyte noncarrier level or neither parent a carrier) has been identified to date. The conclusion

Table 11. Results of Serum Testing in Relatives of Identified TSD Heterozygotes

Relationship to Carrier	Carrier Frequency Expected	No. Tested	Noncarriers	Carriers	Carrier Frequency Observed
Parent	0.50	121	59	62	0.51
Sibling	0.50	52	29	23	0.44

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Table 12. Results of Voluntary Tay-Sachs Screening Program

Population	Results
Subjects tested	6938
Carriers identified	315
"At-risk" couples identified	11

therefore is that serum false carriers (excluding BCP women carriers who should be retested) occur in fewer than 1 in 100 persons designated to be carriers by the serum assay and assignment method used.

Overall Program Results

The overall results of the first year of the J. F. Kennedy Institute Tay-Sachs program are shown in Table 12. Of the 6,938 persons voluntarily tested, 315 carriers were identified in the first year of this program. In addition to those tested in the first community sessions (Table 7), 1200 more persons were tested in another community-screening program or by coming into the program office. Most importantly, 11 couples were identified in whom both husband and wife were carriers of the Tay-Sachs gene, and thereby at-risk for TSD in their offspring. None of these couples had previously had a Tay-Sachs child. In four, a positive family history of TSD existed on one side of the family. A couple is defined as "at-risk" if *both* husband and wife are carriers as shown by the serum-Hex A screening test and by the leukocyte Hex A test. In each of the 11 couples identified, these criteria were met.

Of the 11 identified at-risk couples, 4 already had children. Three of the four couples had two children, and one had one child. None of the children had TSD (two were under 1 year and were tested). Two of the 11 couples were engaged at the time their at-risk status was determined. After intensive counselling, both couples elected to continue their engagement and to be married. They decided to have amniocentesis when pregnancies occurred in the future. Since the time of identification, five pregnancies have occurred in these 11 couples. Each of the pregnancies was monitored by amniocentesis. In one of the pregnancies, there was total absence of hexosaminidase A in the amniotic fluid supernatant, uncultured cells, and subsequently studied cultured cells. The family elected to terminate the pregnancy and studies of the abortus revealed total absence of tissue hexosaminidase A, elevation of GM_2 ganglioside in the cerebral tissues, and ultrastructural evidence of TSD in the fetus (Kaback, M. M., Holtzman, N., Reynolds, L., and Thomas, G., manuscript in preparation). In this family there was no previous history of TSD on either side of the family.

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INFORMING SCREENEES

At the completion of each community-based screening session, blood samples were packaged, returned to the laboratory, and stored overnight at 4°C. Serum samples were then prepared and analyzed. As previously mentioned, the serum from the husbands of pregnant women was analyzed initially and the results immediately telephone-communicated to the families. If a "pregnant husband's" serum level was carrier or inconclusive, the couple was informed of the need for follow-up leukocyte testing for both of them to assess the "still unlikely" possibility of their being an at-risk couple. The remainder of the sera were tested sequentially and no results were conveyed until all samples from the entire testing had been completed. All results were completed within 4 to 8 weeks of the testing date as the screenees had been informed when tested.

After analyses were completed, the questionnaire forms of all inconclusives and carriers were analyzed. Forms were checked for the use of birth control pills, unusual medications, or unusual illnesses. In addition, a possible match-up was looked for between the name and address of any person who was a carrier or inconclusive with any other person (the spouse) in the carrier or inconclusive group. If a couple was carrier-carrier, inconclusive-carrier, or inconclusive-inconclusive, a telephone call was made to the family to explain the situation and to ensure that follow-up leukocyte testings would be performed promptly.

In all other situations, a letter was mailed to each person. If a person was a carrier and his spouse a noncarrier, the carrier was informed by letter also. Each person received a separate letter with the results of his or her test. Because the program dealt with an adult population, absolute confidentiality could be maintained in this way. No one received the results of any test other than his or her own.

Carriers, in addition to getting a form letter, received an explanatory brochure and a detailed instruction pamphlet about the meaning of a carrier status, and its possible implications for other members of the family. Each carrier was instructed to contact the program leaders directly by phone or mail so that further discussion, counselling, and instruction could be carried out. Personalized counselling was achieved in this way or by direct sessions with the carrier if desired.

RELATIVES OF CARRIERS

A relatively complicated part of the program dealt with the question of program responsibility to relatives of identified carriers. Each carrier was informed of the implications of his carrier identification for close relatives

(brothers, sisters, cousins). Accordingly, each carrier was asked to contact the program, and packets of informational material were provided to the carrier for him to convey to the appropriate members of his family. We felt that the program did not have the right to directly contact such relatives without their consent, since this could be regarded as an invasion of privacy. Therefore, this was done through the identified carrier. Before communicating with cousins, each carrier was asked about the possibility of first testing his parents. This, of course, enabled us to ascertain on which side of the family the cousins needed to be contacted. In addition, it enabled us to verify and corroborate the carrier status of the individual. Needless to say, one worried about the situation of a serum and leukocyte-proved carrier in which neither parent proved to be a carrier. This could be the result of a new mutation, but would more likely be due to incorrect paternity or possible mix-ups in the newborn nursery. Fortunately, this situation did not occur in our experience.

When educational packets were conveyed to relatives, even at great distances, the information provided informed the relative as to the nature of the situation and instructed that person to contact the program office in Baltimore. After so doing, arrangements were made with physicians in the area where the relative lived to obtain appropriate serum samples and to send them to the center for testing. Before beginning the program, 75 laboratories across the United States and Canada were contacted, and agreed to obtain, process, and send frozen serum or leukocyte samples from relatives of identified carriers in their area.

This aspect of the program places a major load on the testing center and its office personnel. Each carrier initiates a small "avalanche." On the average each carrier led to approximately 26 additional tests. In addition, the difficulties of communicating with persons, sometimes at great distances, can be formidable. This was recognized, however, as an essential part of the carrier-detection program. The married close relatives of an identified carrier are at a substantially greater risk for the disease in their children. The married sibling of an identified carrier (if married to a Jewish person) has a 1 in 60 chance of being in an at-risk couple ($1/2 \times 1/30 = 1/60$ or 15 times the risk of untested "virgin" Jewish couples). The married cousins (on that side of the family carrying the gene) have a 1 in 120 risk of being in an at-risk couple. Although the administrative problems may be greater in "reaching out into the family," the preventive benefits are assuredly worth it. Three of the eleven at-risk couples identified were reached in this way.

PSYCHOSOCIAL CONSIDERATIONS

Because of the unprecedented nature of voluntary, adult-heterozygote screening, a series of psychosocial studies were initiated with the screening program. As previously mentioned, the major areas of interest with regard to

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RELATIVES OF CARRIERS

A relatively complicated part of the program dealt with the question of program responsibility to relatives of identified carriers. Each carrier was informed of the implications of his carrier identification for close relatives

(brothers, sisters, cousins). Accordingly, each carrier was asked to contact the program, and packets of informational material were provided to the carrier for him to convey to the appropriate members of his family. We felt that the program did not have the right to directly contact such relatives without their consent, since this could be regarded as an invasion of privacy. Therefore, this was done through the identified carrier. Before communicating with cousins, each carrier was asked about the possibility of first testing his parents. This, of course, enabled us to ascertain on which side of the family the cousins needed to be contacted. In addition, it enabled us to verify and corroborate the carrier status of the individual. Needless to say, one worried about the situation of a serum and leukocyte-proved carrier in which neither parent proved to be a carrier. This could be the result of a new mutation, but would more likely be due to incorrect paternity or possible mix-ups in the newborn nursery. Fortunately, this situation did not occur in our experience.

When educational packets were conveyed to relatives, even at great distances, the information provided informed the relative as to the nature of the situation and instructed that person to contact the program office in Baltimore. After so doing, arrangements were made with physicians in the area where the relative lived to obtain appropriate serum samples and to send them to the center for testing. Before beginning the program, 75 laboratories across the United States and Canada were contacted, and agreed to obtain, process, and send frozen serum or leukocyte samples from relatives of identified carriers in their area.

This aspect of the program places a major load on the testing center and its office personnel. Each carrier initiates a small "avalanche." On the average each carrier led to approximately 26 additional tests. In addition, the difficulties of communicating with persons, sometimes at great distances, can be formidable. This was recognized, however, as an essential part of the carrier-detection program. The married close relatives of an identified carrier are at a substantially greater risk for the disease in their children. The married sibling of an identified carrier (if married to a Jewish person) has a 1 in 60 chance of being in an at-risk couple ($1/2 \times 1/30 = 1/60$ or 15 times the risk of untested "virgin" Jewish couples). The married cousins (on that side of the family carrying the gene) have a 1 in 120 risk of being in an at-risk couple. Although the administrative problems may be greater in "reaching out into the family," the preventive benefits are assuredly worth it. Three of the eleven at-risk couples identified were reached in this way.

PSYCHOSOCIAL CONSIDERATIONS

Because of the unprecedented nature of voluntary, adult-heterozygote screening, a series of psychosocial studies were initiated with the screening program. As previously mentioned, the major areas of interest with regard to

these studies are (1) the effectiveness of the educational program, (2) the factors that relate to compliance and noncompliance with a voluntary test of this nature, and (3) the possible impact or stigmatization of carrier identification on the individual, the couple, or the family.

Many of these studies are still in progress at this time. For obvious reasons certain evaluations (particularly the impact studies) will take a substantial period of time for adequate assessment to be made. However, it is of interest to look at the initial studies regarding compliance and educational effectiveness.

As previously indicated, detailed questionnaires were self-administered by all participants at the time of testing. A series of persons exposed to an identical educational program, but who had not presented for testing, were mailed a similar questionnaire. More than 80 percent of a randomly selected group of 500 nonparticipants completed the questionnaires and returned them to the program. A detailed comparison was then conducted between these two samples. The in-depth analysis of these data will be reported elsewhere (Kaback et al., 1973). It is sufficient, however, to point out that critical analysis of these data indicated that the differences were significant (at least $p < 0.05$) between the participant and nonparticipant groups. Respondents were significantly younger, had fewer children, were less likely to have completed their families, were more educated, and were higher on the social index scale. In addition, and perhaps most importantly, by several criteria, the participants appeared to understand more clearly the genetic information regarding carrier risk and implications and the subsequent alternatives if they alone or with their spouses were found to be heterozygotes.

The religious attitudes, the perceived health status of the person, drug use, and attitudes toward abortion did not differ significantly between the participants and nonparticipants. There were significant differences between the groups in their comprehension of the genetic information, the perceived susceptibility of being a carrier, and the implications of being a carrier. It would appear from these data that voluntary heterozygote screening (for conditions with positive reproductive alternatives, i.e., amniocentesis for at-risk families) most appeals to younger couples planning future children. This incentive may be the primary stimulus for intensive evaluation and understanding of the genetic information provided. Needless to say, this has important implications for future genetic screening programs in which mass education and voluntary response would be applied.

Studies to assess the effectiveness of genetic counselling and the impact of carrier detection on individual carriers and their families are currently being conducted. As one might anticipate, a significant time period may be necessary to appreciate significant effects of counselling or possible carrier stigmatization in such a study. These determinations, however, may be the most essential in evaluating this approach as a prototype for prospective genetic disease prevention.

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GENETIC CONSIDERATIONS

The implications of widespread heterozygote screening in a selected population, and the provision of a mechanism for reproduction, even in at-risk couples, raises several questions about possible effects of such a program on the gene frequency in that population. It might appear that providing a mechanism for couples, in which both members are heterozygous, to reproduce freely would cause a significant increase in the gene frequency (since each child they would now bear would have a two-thirds chance of being heterozygous). However, if one makes several, not unreasonable assumptions about reproductive behavior in this population in the future, it is evident that heterozygote screening for an autosomal recessive gene like the Tay-Sachs gene would have relatively little impact on the gene frequency in the subpopulation involved (Kaback and O'Brien, 1971).

Since the great majority of heterozygous persons reproduce with nonheterozygous persons, the major proportion of heterozygotes per generation is derived from these matings. The relatively rare double heterozygous matings now having offspring would increase the number of heterozygotes per generation only slightly from what it might otherwise have been. This is illustrated in Table 13. A simple calculation, with the Tay-Sachs program in mind, indicates that if all Ashkenazi Jews of child-bearing age were screened for the Tay-Sachs gene, and all couples were able to have two children (none with Tay-Sachs disease), the overall affect would be a "relative increase" in the gene frequency of 0.019 per 25-year generation. At

Table 13. Genetic Implications of Tay-Sachs Screening Program

Test Population	Without Program	With Program
Expected births to defined population per 25-year generation	1.5×10^6	1.5×10^6
Children of AA × AA matings (700,850)	1,401,700 AA	1,401,700 AA
Children of AA × Aa matings (48,300)	48,300 AA	48,300 AA
	48,300 Aa	48,300 Aa
Children of Aa × Aa matings (850)	425 AA	567 AA
	850 Aa	1,134 Aa
	425 aa	—
Totals	1,450,425 AA	1,450,567 AA
	49,150 Aa*	49,433 Aa
	425 aa	—

Note: Assumptions: (1) North American Jewish population = 6×10^6 subjects; (2) 1.5×10^6 subjects were 18 to 45 years old in this population; (3) Zero population growth (2 children per couple); (4) No reproductive compensation.

*In one generation: Initial total carriers = 50,000, resultant total carriers = 49,150.

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this rate, thousands of years would be necessary for a significant change in the TSD heterozygote frequency to be evident in Ashkenazi Jews as the result of such a program.

ECONOMIC AND SOCIAL CONSIDERATIONS

Although the genetic hazards of the screening program seem minimal, what of the economic feasibility and the social impact of such an effort? Analysis of the program described indicates that about $4.00 in costs are incurred for each person screened. (See Table 14.) This assumes, of course, a program of sufficient size to make testing maximally efficient. Parenthetically, this would speak for regionalizing such screening programs, if projected on a national scale, so that unnecessary laboratory duplications are avoided. It may be possible to minimize the costs further by reducing the number of persons requiring the leukocyte retest ("inconclusive" women on

Table 14. Tay-Sachs Screening Program Costs*

1. Costs are calculated on basis of testing 50,000 people over 3 years.
2. Costs include costs of leukocyte retesting in approximately 6 percent of all subjects screened (mainly serum inconclusives and females on BCP).
3. Costs calculated by assuming each person's serum will be assayed an average of two times.
4. Excludes professional salary, overhead, depreciation, etc.

Program costs for 3 years

Equipment	30,000
Technicians (3)	81,000
Coordinator and secretary	50,000
Lab and office supplies	15,000
Printing, mailing, etc.	10,000
	$186,000
Retesting (WbC) costs—3,000 @ $3.00 per	9,000
	$195,000

$195,000 ÷ 3 years = $65,000/year

240 work days/year; 200 assays/day × 150 days = 30,000 tests/year or 15,000 people screened annually

$$\frac{\$65{,}000}{15{,}000 \text{ persons}} = \$4.35 \text{ per person screened}$$

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Table 15. A National Tay-Sachs Screening Program* (Cost/Effectiveness Analysis)

Service	Subjects	Costs
Screening tests	1.5×10^6 18 to 43-year-old married subjects @ $4.00	$\$6.0 \times 10^6$
Amniocentesis, cell testing	850 couples; 2267 pregnancies @ $100.00	$\$0.23 \times 10^6$
Abortions	567 pregnancies @ $200.00	$\$ .12 \times 10^6$
Total cost of program		*$\$6.35 \times 10^6$*
Average medical cost/year/child (over 3.5 years)	425 children with Tay-Sachs disease (life expectancy: 3.5 years)	$15,000.00
Average lifetime medical costs/child		$52,500.00
Total cost of 425 children		*$\$22.3 \times 10^6$*

*If population remains stable and other assumptions are correct.

birth control pills with high total serum hexosaminidase activity and a percent Hex A greater than 47 percent), or by *totally* automating the assay procedure (Lowden, J. S., Skomorowski, M. A., Henderson, F., and Kaback, M. M., 1973).

An overall cost/effectiveness analysis of such a screening and prevention program (with a North American population of 6×10^6 Jewish persons) is shown in Table 15. This indicates, even at current costs, that the prospective prevention of those relatively rare cases of TSD in the high-risk population would cost far less than the amount necessary to meet the medical/hospital costs generated by the care of children with TSD who would otherwise be born. Such a consideration says nothing, of course, of the major costs—the "human" ones—incurred by such tragic illnesses.

This method for the control of TSD might be considered an interim approach. Certainly, an effective therapy (or even cure) for the disease could be viewed by some as a more attractive means to control this disorder. Although much is known about the underlying mechanism of this condition, all attempts at correcting the metabolic error (enzyme replacement, etc.) have been unrewarding thus far (O'Brien, 1973; Kaback, unpublished observations). Until such an approach is developed, therefore, it would seem that the screening-prevention program can serve as a reasonable, albeit imperfect, alternative.

In the first year of the Maryland program, approximately 7,000 persons volunteered for this test. To date, a total of nearly 10,000 have been screened. Although the approximate number of Jewish persons of child-bearing age

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(age 18 to 43 years) in that region is nearly 60,000, the actual compliance is hard to ascertain. More than 85 percent of those volunteering for testing indicated plans for having more children (as compared with less than 20 percent of those not choosing to be tested). This suggests that the compliance calculation should use as its denominator, not 60,000, but rather the total number of persons planning future reproduction. Accordingly, as many as one-third of the total persons who might "directly benefit" by being screened may already have complied. This may compare favorably with other voluntary health programs. A further increase in this "true" compliance ratio may occur as additional community testings are conducted (as are planned).

CONCLUSIONS AND IMPLICATIONS

Heterozygote screening in healthy adult populations raises new and important questions for the medical geneticist, for the social scientist, for persons in the involved community, and in many ways for the society at large. Genetic medicine and the simplest concepts of human genetics are abstractions for the public at this time. Hopefully, through formal and informal educational mechanisms, this will be changed in future years. Currently, however, the delicate and possibly even threatening aura of this "new health message," and the complex social and ethical issues involved, make voluntary heterozygote screening and prospective genetic disease prevention intricate and, at times, controversial matters (Harris, et al., 1971; Lappé et al., 1972; Hilton et al., 1973).

In light of these innovations, the traditional role of the medical geneticist is changed. In the past, medical genetics has been a respondent discipline, reacting primarily to the proband-related needs of patients and families referred for services and counselling. With the advent of large-scale heterozygote screening programs, the geneticist adopts a new role—that of an initiator, or health advocate who seeks out the public at-large, informs and educates, and then must deal with a more abstract-type of counselling (nonproband related) on a relatively enormous scale.

Clearly, the need for rigorous inspection and evaluation of pilot programs is essential. The data and experiences accrued from early screening studies should have important implications for heterozygote detection programs of the future. Certainly, the potential complexities of voluntary screening of adult populations for other common recessive genes such as cystic fibrosis, thalassemia, and sickle cell, may be minimized by the experiences gained with the early prototype programs.

In summary, the first major effort to deliver a voluntary, adult-oriented, heterozygote screening program to a defined community is described. With

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institution of a semiautomated method for serum hexosaminidase A quantitation, and back-up leukocyte determinations in selected persons, an accurate genotype designation can be made in at least 99 percent of the screened population. To date, no known false-positives or false-negatives have been identified. An overall carrier frequency of approximately 1 in 24 persons tested was determined in the Jewish populations of Baltimore, Maryland and Washington D.C. In the first year of the program 11 couples, none of whom had previously had a Tay-Sachs child, were identified to be at-risk for TSD in their offspring. In one couple, with no history of TSD on either side of the pedigree, a fetus with TSD was identified in utero, electively aborted, and the diagnosis confirmed in aborted fetal tissues.

Although the technological aspects of heterozygote screening are reasonably straight-forward, the complexities of program design and delivery are substantial. With a strategic educational program, the community involved was integrated into the program at all levels—support, planning, and delivery. This concept perhaps was most responsible for the apparently favorable response the program received from various segments of the community.

It appears that heterozygote screening on a voluntary basis for disorders with positive reproductive alternatives most appeals to young married couples with future plans for children. In this group, it appears that an intensive educational campaign can be effective in eliciting a meaningful response from the community.

Although the genetic implications of mass-screening for autosomal recessive genes appears to be minimal, the social and ethical concerns may be substantial. Accordingly, the essential role of social scientists in the evaluation of pilot screening programs is underscored.

Voluntary heterozygote screening in adult populations has been developed (sickle cell) or is likely to develop for the prevention of other recessive diseases (thalassemia, cystic fibrosis, etc.). The principles, issues, and psychosocial considerations addressed in this prototype Tay-Sachs disease prevention effort should have important and relevant implications for such programs.

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6

The XYY Chromosome Male—or Syndrome?

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INTRODUCTION 137
Historical Background 137
Evidence for an Association between XYY and Antisocial Behavior 138
Is There an XYY Syndrome? 141
The Critical Role of Population Sampling 142
CYTOGENETICS STUDIES 144
The Y Chromosomes 144
Simultaneous Occurrence of 47,XYY with Other Trisomies 147
POPULATION SURVEYS 148
General Population 148
Special Populations 149
Parental Ages at Birth and Nondisjunction 151
Distribution by Ethnic Origin 151
PHYSICAL AND MEDICAL CHARACTERISTICS 153
Height and Weight 153
Skeletal Features 156
Dermatologic Features 157
Cardiac Involvement 158
Dermatoglyphics 159
Endocrine Studies 160
Meiosis and Reproduction 161
Neurological Studies 163
A Cerebral Pathology Hypothesis 165
BEHAVIORAL CHARACTERISTICS 169
Some Introductory Remarks 169
Psychologic and Psychiatric Findings 172
Intelligence 172
Personality 175

This work was financially supported in part by USPHS grants MH 17565 and MH 19337.

Psychiatric History and Characteristics 178
Family Background and Stability 178
Mental Illness and Subnormality 179
Treatment Considerations 181
Social and Criminological Data 185
Family and Social Class Factors 186
Antisocial and Criminal Behavior 187
Deviant Behavior and Societal Response 192
SOME LEGAL AND SOCIAL IMPLICATIONS 195
Criminal Law and Related Issues 195
The XYY Anomaly and the Insanity Defense 195
Special Handling and Preventive Detention 197
Criminal Sentencing and Parole Decisions 198
Some Social Policy Considerations 199
Issues of Confidentiality and Informed Consent 199
Issues Relating to Genetic Counseling 199
CONCLUSIONS 201
Summary of Major Findings 201
Some Needs and Implications for Future Research 203
Final Comments 205
REFERENCES 206
APPENDICES 220

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INTRODUCTION 137
Historical Background 137
Evidence for an Association between XYY and Antisocial Behavior 138
Is There an XYY Syndrome? 141
The Critical Role of Population Sampling 142
CYTOGENETICS STUDIES 144
The Y Chromosomes 144
Simultaneous Occurrence of 47,XYY with Other Trisomies 147
POPULATION SURVEYS 148
General Population 148
Special Populations 149
Parental Ages at Birth and Nondisjunction 151
Distribution by Ethnic Origin 151
PHYSICAL AND MEDICAL CHARACTERISTICS 153
Height and Weight 153
Skeletal Features 156
Dermatologic Features 157
Cardiac Involvement 158
Dermatoglyphics 159
Endocrine Studies 160
Meiosis and Reproduction 161
Neurological Studies 163
A Cerebral Pathology Hypothesis 165
BEHAVIORAL CHARACTERISTICS 169
Some Introductory Remarks 169
Psychologic and Psychiatric Findings 172
Intelligence 172
Personality 175

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Psychiatric History and Characteristics 178
Family Background and Stability 178
Mental Illness and Subnormality 179
Treatment Considerations 181
Social and Criminological Data 185
Family and Social Class Factors 186
Antisocial and Criminal Behavior 187
Deviant Behavior and Societal Response 192
SOME LEGAL AND SOCIAL IMPLICATIONS 195
Criminal Law and Related Issues 195
The XYY Anomaly and the Insanity Defense 195
Special Handling and Preventive Detention 197
Criminal Sentencing and Parole Decisions 198
Some Social Policy Considerations 199
Issues of Confidentiality and Informed Consent 199
Issues Relating to Genetic Counseling 199
CONCLUSIONS 201
Summary of Major Findings 201
Some Needs and Implications for Future Research 203
Final Comments 205
REFERENCES 206
APPENDICES 220

INTRODUCTION

Historical Background

The first report of a man with a 47,XYY karyotype was that by Sandberg and colleagues (1961). This man had been studied because he had a child with Down's syndrome. There were other scattered reports of a few more persons with this karyotype, and investigators thought that the endocrine and sexual development were somewhat abnormal in these men *and women* (Balodimos et al., 1966; Boczkowski, 1969).

We note here that, although most of the literature concerning the XYY chromosomal anomaly refers to phenotypic males, there have been a few reports of females with such karyotypes (Boczkowski, 1969; Court Brown, 1968; Franks, et al., 1967; Vignetti, et al., 1964). Most of the cases of the female phenotypes with XYY chromosome complements described in the literature are demonstrable mosaics (45X/47,XYY). In such cases the 45,X line of cells dominates and provides the female phenotype. However, Court Brown (1968) reported on a mentally retarded female in whom extensive chromosome studies on lymphocytes, marrow cells, and fibroblasts from the skin showed a single line of cells with a 47,XYY complement.

Court Brown (1968) has pointed out that knowledge about the XYY chromosome constitution has proceeded through three phases. The first phase was the fortuitous discovery of the aberration while examining male patients with various physical abnormalities. The observation that there appeared to be certain phenotypic characteristics led to the population studies of the second phase.

The second phase was characterized by intensive efforts to locate XYY men in various apparently "high risk" subpopulations, for example, penal and mental institutions and maximum security hospitals. A variety of surveys of special facilities were then undertaken and, as expected, relatively high frequencies of XYY men were often encountered. The case-finding efforts of the second phase are still continuing, as are periodic reports on individual cases.

Concurrently, a third phase had been in evidence since 1968, when various investigators began work on the more important and basic issues (Borgaonkar, 1969): What are the incidence and prevalence rates for the 47,-XYY constitution in the general population? Is the karyotype the only characteristic feature or are there also certain physical, endocrinological, and behavioral characteristics by which such persons could also be identified? What generally can be predicted about a person with an XYY karyotype?

Now that the early dramatic findings and some of the accompanying premature speculations have fallen into perspective and research interest has been stimulated on this broad topic, a fourth phase appears to be developing. Building on earlier work and learning from previous efforts as well as

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