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Medical Genetics

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Medical Genetics

Volume IV

Edited by

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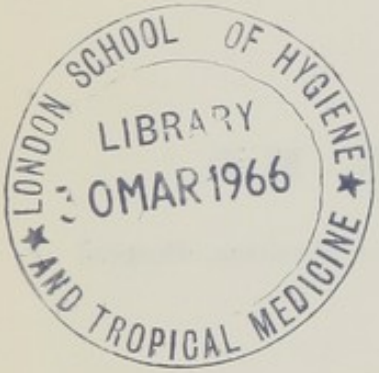

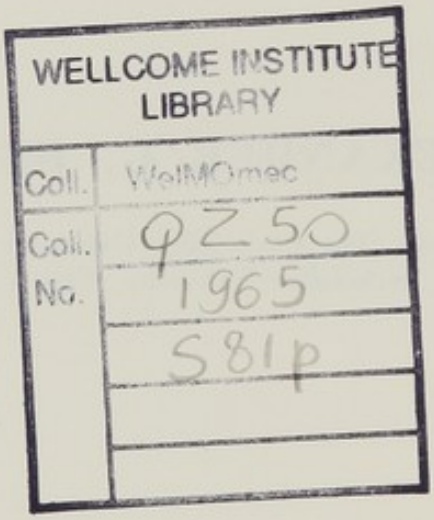

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Contents

Foreword

THE FOURTH VOLUME of Progress in Medical Genetics like its predecessors, contains critical and selective reviews of several topics which we hope will be of interest not only to those working in human genetics, but also to those physicians who recognize the increasing importance of the role of heredity in our understanding of health and disease.

The opening chapter by R. D. A. Peterson, M. D. Cooper and R. A. Good on disorders of the thymus and other lymphoid tissues emphasizes the key role that genetics must play in any attempt to understand the complexities of immunologic disease. The need for an understanding of both genetics and biochemistry in the clinical syndromes characterized by abnormal glycogen storage, is amply demonstrated by J. B. Sidbury's discussion of this subject in Chapter 2. The difficult problem of congenital malformations is discussed in Chapter 3 by C. O. Carter. A uniquely comprehensive and critical review of the evolutionary origins of human proteins by C. J. Epstein and A. G. Motulsky (Chapter 4) stresses the evolutionary aspects of human proteins and indicates how comparative structural studies on homologous proteins from different species can illuminate our understanding of human evolution. The discovery that enzymes can exist in multiple molecular forms with similar substrate specificities has opened up a new field of great relevance to our understanding of the genetic control of protein structure. E. S. Vesell in Chapter 5 has discussed these relationships with particular reference to lactic acid dehydrogenase. A. C. Allison and B. S. Blumberg in Chapter 6 give a valuable account of the serum lipoprotein allotypes in man, a field which is already expanding rapidly to include immunologic variations in other serum proteins. South East Asia and Australasia comprise a region with a rich variety of culturally and biologically differentiated populations suitable for genetical investigation. The aborigines of Australia living at a pre-Neolithic level of economy in a vast continent in which they have presumably been relatively isolated for some thousands of years have long excited the interest of anthropologists and Dr. R. L. Kirk's summary in Chapter 7 of his own work and of the scattered genetical data is particularly welcome. The closing chapter by G. Allen (Chapter 8) on the use of twins in human genetics is an extremely valuable corrective for those who think that it is easy to apportion the relative contribution of genes and environment to the phenotype by means of twin studies.

The debt of the editors to the authors is considerable. It is no easy task to find time to write a critical and scholarly review in the midst of the hurly-burly of research. But in so doing, the authors have rendered a real service to those who still try to keep abreast of recent advances in human genetics.

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

Chapter 1

Disorders of the Thymus and Other Lymphoid Tissues

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It has become increasingly evident over the last several years that immunologic deficiency states, "autoimmune" diseases, and lymphoid malignancies are interrelated pathogenetically: two or more of these conditions often occur simultaneously in a single patient and, in addition, a predisposition to these disturbances exists in some families. Even more recently, as some of these problems have been taken into the laboratory, evidence has been brought forward suggesting that common disturbances of the thymus and other lymphoid tissues may underlie all three, both as they occur clinically and as they are observed in experimental animals.

It is the purpose of this review to bring together some of the widely scattered clinical and experimental evidence supporting this hypothesis. This task is facilitated by the recent growth in our knowledge concerning the lymphoid tissue. The latter is both the effector of the immune

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response and the tissue involved by the malignancies to be discussed. Present evidence suggests that the lymphoid tissue of higher vertebrates is composed of two specialized cellular components, each with its own origin and morphologic and functional characteristics (Cooper, Peterson and Good, 1965; Sutherland et al., 1964). Because this concept is new and relevant to the consideration of disorders of the lymphoid tissues, the first section of this review will define and briefly present the evidence for this concept.

Development of the Lymphoid Tissue

All the evidence to date indicates that the lymphoid cells of the body have their anlagen in tissues that are either directly derived from the gut epithelium or at least closely associated with it (Auerbach, 1960; Ackerman and Knouff, 1959; Archer, Sutherland and Good, 1964). We will refer to such primordial tissue as "central lymphoid tissue" and to the other lymphoid tissue, present for instance in the spleen and lymph nodes, as "peripheral lymphoid tissue."

Direct evidence in the chicken (Cooper, Peterson and Good, 1965) and less direct evidence in rabbits (Sutherland et al., 1964) indicates that there are two distinct types of peripheral lymphoid tissue, each derived from and dependent upon a different central lymphoid tissue. One of the forms of peripheral lymphoid tissue, populated primarily by small lymphocytes, has its major anlage in the thymus, is nurtured by the thymus, and can be considered the "thymus-dependent lymphoid tissue." The other exists in all higher vertebrates studied, but has its primordial tissue defined at present in only one species, the chicken (Glick, Chang and Jaap, 1956; Mueller, Wolfe, Meyer and Aspinall, 1962). In the chicken a hind-gut lymphoid organ, the bursa of Fabricius, is the central lymphoid organ for peripheral lymphoid tissue composed primarily of large pyroninophilic lymphocytes and plasma cells (Warner, Szenberg and Burnet, 1962; Isaković and Janković, 1964; Cooper, Peterson and Good, 1965). Since mammals lack a bursa but have tissue comparable to the bursa-dependent tissue of chickens, it is perhaps most convenient to denote this type of peripheral lymphoid tissue in functional terms, the "immunoglobulin-producing system." Studies of the developing lymphoid tissues can be classified into three categories, phylogenetic, embryologic and ablation experiments, and will be discussed in that order.

Phylogenetic studies suggest that a major portion of the lymphoid cell system has its anlage in the thymus, a gut-derived organ. The hagfish, perhaps the most primitive vertebrate form, lacks all lymphoid tis-

sue including a thymus, and also lacks an immune system definable by classic immunologic methods (Good and Papermaster, 1961; Papermaster, Condie and Good, 1962; Papermaster, Condie, Finstad and Good, 1964). In the lamprey, a slightly more advanced form phylogenetically, is observed a thymus primordium represented by foci of lymphoid cells in the epithelium of the pharyngeal gutters; clusters of lymphoid cells in the spleen; blood lymphocytes; and the capacity to develop delayed allergy, produce small amounts of antibody against a few of many antigens, reject skin homografts, and form small amounts of gamma globulin (Papermaster, Condie, Finstad and Good, 1964; Finstad, Papermaster and Good, 1964; Finstad and Good, 1964). In higher, but still primitive, fishes such as the holosteans and chondrosteans, there are true thymic tissues in the gill areas and more advanced lymphoid structures in the gut and anterior kidneys, correlated with greater immunologic potential particularly for antibody production (Finstad, Papermaster and Good, 1964; Good and Finstad, 1964; Papermaster, Condie, Finstad and Good, 1964). These higher fishes have a plasma cell system, the specialized immunoglobulin-producing system not found in lower fishes like the lamprey.

Embryologic studies of the development of the thymus-dependent lymphoid cell system have been carried out in several species. Auerbach (1960, 1961, 1963, 1964) demonstrated that in the mouse gut-epithelial cells in the region of the second to fifth pharyngeal pouches differentiate and become the lymphoid cells characteristic of the mature thymus. Mesenchyme is required for the differentiation of epithelial to lymphoid cells, but, contrary to earlier belief, mesenchymal cells are not the precursors of the early thymic lymphocytes.

The thymus is the first organ to become lymphoid in all species that have been studied, and it remains the only lymphoid organ while the stromal elements of the peripheral lymphoid tissues such as spleen and lymph nodes develop (Sabin, 1909; Archer, Kelly, Papermaster and Good, 1963; Block, 1964; Good and Papermaster, 1964). The thymus-dependent lymphoid tissue is probably best visualized in rabbits thymectomized and then given sufficient irradiation to destroy most of the peripheral lymphoid tissue already present. While nonthymectomized-irradiated animals show complete lymphoid tissue recovery, the thymectomized-irradiated animals show long-term depletion of their thymus-dependent peripheral lymphoid tissue (Sutherland et al., 1964). In such animals the thymus-dependent lymphoid tissue is evidenced by its absence. It comprises much of the small lymphocyte population of the body including most of the blood lymphocytes and especially the

collars of small lymphocytes that surround the germinal centers of lymphoid follicles in the lymph nodes, spleen, and wherever else follicles are found. The germinal centers and plasma cells remain since they are not thymus-dependent but rather are part of the immunoglobulin-producing system whose central lymphoid tissue is not the thymus.

It is known that the rabbit appendix also contains lymphoid tissue that seems to function as an important source of what we now view as thymus-dependent lymphoid tissue (Archer, Sutherland and Good, 1963, 1964). This issue needs further study.

There is evidence for two mechanisms of thymic influence on the development of the animal's lymphoid tissue: actual population of the peripheral lymphoid tissues by thymic lymphocytes (Harris and Ford, 1964; Nossal and Gorrie, 1964; Sainte-Marie and Leblond, 1963) and by a humoral substance released by the thymus (Osoba and Miller, 1964; Levey, Trainin and Law, 1963).

Extensive experimental work has established that in the rabbit (Archer and Pierce, 1961; Archer, Pierce, Papermaster and Good, 1962), mouse (Miller, 1961a; Good et al., 1962), rat (Arnason, Janković, Waksman and Wennersten, 1962; Janković, Waksman and Arnason, 1962), and hamster (Sherman, Adner and Dameshek, 1964; Defendi, Roosa and Koprowski, 1964), removal of the thymus early in development interferes with establishment of full immunologic capacity. The most critical studies have been performed in the mouse. These experiments have shown that neonatal thymectomy interferes with development of immunologic capacity, including homograft immunity (Miller, 1961a; Good et al., 1962), capacity of spleen cells to exercise graft versus host reactions (Good et al., 1962) and formation of circulating antibodies, especially against certain weak antigens (Papermaster, Dalmasso, Martinez and Good, 1962; Miller, 1963; Humphrey, Parrott and East, 1964). In addition, neonatally thymectomized mice are inordinately susceptible to infection with a variety of organisms (Salvin, Peterson and Good, 1965; Hadley and Elberg, 1964) and to the lethal effects of endotoxin (Salvin, Peterson and Good, 1965). Further, neonatally thymectomized mice regularly develop runting, wasting, and die at an early age (Miller, 1961a; Good et al., 1962).

Thus, animals deprived of their thymus-dependent lymphoid tissue have varying degrees of immunologic deficiency affecting delayed hypersensitivity, transplantation immunity, and the production of specific serum antibodies. Paradoxically, such animals may have normal or increased numbers of plasma cells, and normal levels of serum immunoglobulins, and may respond well to stimulation with strong antigens

(Janković, Waksman and Arnason, 1962; Archer, 1964; Humphrey, Parrott and East, 1964).

Recent studies in chickens suggest that it may be helpful to consider the thymus-dependent cell system as that responsible for the recognition phase of the immune response, capable of recognizing foreignness of antigens, mediating cellular (delayed type) hypersensitivity, and perhaps passing this information, by some mechanism of cell-cell communication, to the immunoglobulin-producing system where the bulk of specific antibodies as well as the immunoglobulins are formed.

Development of the Immunoglobulin-Producing System

Large lymphoid cells of the germinal centers, transitional cells, and plasma cells are the principal producers of antibodies and immunoglobulins (Kolouch, 1939; Bjørneboe and Gormsen, 1943; Fagraeus, 1948; Coons, Leduc and Connolly, 1955; Ortega and Mellors, 1957; Vazquez, 1958; Curtain and O'Dea, 1959; Mellors and Korngold, 1963). These cells apparently do not develop from the thymic primordium, but have a distinct developmental pattern and probably a lineage of their own from other central lymphoid tissue. At present the chicken affords the best experimental model for identifying the immunoglobulin-producing system and for distinguishing it from the thymus-dependent lymphoid tissue (Cooper, Peterson and Good, 1965). Nonetheless, rapid progress along these lines is being made in mammals as well. The immunoglobulin-producing system of the chicken has its anlage in the bursa of Fabricius. Removal of the bursa from a newly hatched chicken markedly impairs the capacity of these birds to produce serum antibodies to later antigenic stimulation (Glick, Chang and Jaap, 1956; Mueller, Wolfe and Meyer, 1960). It does not apparently impair the animal's ability to develop delayed hypersensitivity (Janković, Išvaneski, Milošević and Popesković, 1963; Cooper, Salvin, Peterson and Good, 1964). To clearly delineate what is bursa-dependent and what is thymus-dependent lymphoid tissue, we found it necessary to irradiate the newly hatched chicken to eliminate lymphoid tissue already established in the periphery by the time of hatching. In such birds, two types of lymphoid tissue can be clearly distinguished. Bursa-dependent lymphoid tissue consists of large lymphocytes located in very distinctive splenic follicles, plus the plasma cells. These lymphoid elements are clearly distinguishable from the thymus-dependent white pulp. The plasma cells and the cells of the bursa-dependent follicles are the immunoglobulin producing cells. Chickens bursectomized and irradiated in the newly hatched period have both 19S and 7S agammaglobulinemia, and are completely un-

able to produce circulating antibodies in response to an antigenic stimulus (Cooper, Peterson and Good, 1965). As would be anticipated, however, bursectomized-irradiated birds have intact their thymus-dependent lymphoid tissues, and we have already shown that they can develop and mediate certain forms of cellular immunity (Cooper, Schwartz, Peterson and Good, 1964). According to our present concept, it is the thymus-dependent recognition system working together with the bursa-dependent immunoglobulin-producing system that constitutes the immune system of cells. An intimate and as yet undefined relationship, perhaps involving transmission of information between these two systems, must exist to account for the production of specific antibodies. The central lymphoid organ or organs of the mammalian immunoglobulin-producing system equivalent to the chicken bursa have not yet been defined, but that such a distinct system exists is strongly indicated by the studies cited previously of mammals deprived of their thymus-dependent lymphoid tissues. In the latter the germinal center component and plasma cells, as well as capacity to form immunoglobulins, seem entirely intact.

"Autoimmune" Phenomena in Experimental Animals

Our discussion of "autoimmune" phenomena or diseases is probably best prefaced with a brief comment on the sense in which we accept this term as a useful one. It characterizes the presence in an animal's serum of antibodies that react with various tissues and substances from its own body, but does not necessarily imply that the autoantibodies are pathogenetic in the often associated diseases. For purposes of this review, autoantibodies will be considered indicators of a disturbed immune system, leaving aside entirely the question of whether they are primary, secondary, or entirely incidental to the disease process with which they are associated.

Perhaps one of the more provocative examples of autoimmune phenomena in experimental animals is their occurrence in animals deprived of their thymic-dependent lymphoid tissue. Rabbits subjected to neonatal thymectomy or neonatal thymectomy-appendectomy often develop Coombs positive red blood cells and amyloidosis or preamyloid changes, but runting and wasting rarely occur (Sutherland et al., 1965). This syndrome also occurs in adult rabbits subjected to thymectomy or thymectomy-appendectomy, followed by 500 r of irradiation (Kellum et al., 1965) and is usually accompanied by runting and wasting, and occasionally by anemia. While control irradiated rabbits usually recover completely, these manipulated rabbits continue to have depressed peripheral

lymphocyte counts and abnormal spleens and lymph nodes. These organs tend to be deficient in small lymphocytes and in follicular development, while they have massive accumulations of reticulum cells and plasma cells. This paradoxic association of immunologic deficiency and autoimmune phenomena in rabbits parallels clinical observations (see below).

deVries et al. (1964 a,b) have observed autoimmune phenomena in certain strains of inbred mice after neonatal thymectomy. These animals develop wasting, have Coombs positive red blood cells, L.E. rosettes, and L.E.-like lesions in the kidney and heart.

Autoimmune phenomena also occur spontaneously in certain strains of mice apparently on a genetic basis. This is most important in NZB and especially in (NZB X NZW)F_1 mice (Bielschowsky, Helyer and Howie, 1959; Holmes, Gorrie and Burnet, 1961; Helyer and Howie, 1963; Burnet and Holmes, 1962). These animals develop a Coombs positive hemolytic anemia, antinuclear factors, lupus-like kidney lesions and amyloid disease. In addition, they have an abnormal thymus characterized by germinal lymphoid follicles and plasma cells, structures foreign to a normal thymus. Early thymectomy does not protect such animals from the disease process but transplantation of their thymus can transmit the disease to an ordinarily nondiseased strain.

Taken together, these various experimental models reinforce our contention that autoimmune phenomena may be essentially side effects of either induced or genetic abnormalities in the lymphoreticular system, and more specifically abnormalities in the relationship of the thymic-dependent recognition system and the immunoglobulin producing system.

Experimental Studies of Lymphoreticular Malignancies

Malignancies of the lymphoreticular tissues occur in several animals and provide models in which to study the relationship of lymphomas to developmental and acquired defects of the lymphoreticular tissues. Several strains of mice develop lymphatic leukemia (Gross, 1961a); in some it occurs spontaneously and with great frequency (Slye, 1931; MacDowell and Richter, 1935; Furth, Seibold and Rathbone, 1933), but in others it seldom occurs unless the animal is exposed to a leukemia-inducing virus (Gross, 1951; Gross, 1961b; Gross, 1962; Moloney, 1964) or some other oncogenic agent such as x-ray (Krebs, Rask-Nielsen and Wagner, 1930; Kaplan and Brown, 1952) or chemicals (Burrows and Cook, 1936; Furth and Furth, 1938; Engelbreth-Holm and Lefevre, 1941). Under almost all these circumstances, however, the lymphatic leukemia appears to start in the thymus and, probably as a consequence, thymectomy prior to the onset of the disease will generally prevent the

disease from developing (McEndy, Boon and Furth, 1944; Miller, 1961b). Reimplantation of a newborn thymus into such a mouse will recreate the circumstances necessary for leukemogenesis (Miller, 1962). These mouse lymphocytic malignancies appear to involve exclusively the thymus-dependent lymphoid tissue. Kaplan, Axelrad and others have emphasized that only relatively undifferentiated lymphocytes are susceptible to oncogenic agents and that in the mouse such cells are especially prevalent in the thymus (Carnes and Kaplan, 1956; Kaplan, 1960; Axelrad and van der Gaag, 1962; Dawe, Law and Dunn, 1959; Ham et al., 1960). Another possible explanation for the important role of the thymus in mouse lymphatic leukemia is suggested by the experiments of Metcalf (1956, 1962). He has been able to demonstrate a lymphocyte stimulating factor, apparently produced or concentrated in the mouse thymus, capable of causing proliferation of lymphocytes under certain experimental circumstances. Strains of mice with a high spontaneous incidence of lymphatic leukemia have higher concentrations of lymphocyte stimulating factor in their thymus and blood than do strains with a very low incidence of lymphatic leukemia.

In an effort to further define the role of the central lymphoid tissue in leukemogenesis, we studied the effect of thymectomy and bursectomy on the development of the generalized lymphocytic malignancy of the chicken, visceral lymphomatosis. Chickens infected shortly after hatching with a virus-containing tumor filtrate will quite regularly develop visceral lymphomatosis 5 to 9 months later (Burmester, Fontes and Walter, 1960). We found that surgical removal of the bursa any time up to 2 months of age will almost completely prevent the lymphoma from developing (Peterson et al., 1964; Peterson et al., unpublished observations). Unlike the situation in the mouse, thymectomy alone has no effect on the incidence of visceral lymphomatosis in birds infected when newly hatched. Visceral lymphomatosis is apparently a malignancy involving only the bursa-dependent lymphoid tissue.

Another relationship can also be demonstrated between the immune system and lymphocytic malignancies. A malignancy of the thymus-dependent lymphocytes can be demonstrated to impair the immune response long before the malignancy becomes clinically or histologically demonstrable. Mice inoculated at birth with passage A virus regularly develop lymphatic leukemia approximately 4 months later. The capacity of such mice to produce antibodies to a bacteriophage is impaired as early as 3 weeks after inoculation of the oncogenic virus (Peterson, Hendrickson and Good, 1963). Their homograft immunity is also deficient (Dent, Peterson and Good, 1965). Other carcinogens such as

methylcholanthrene also depress the immunologic responsiveness of an animal prior to the onset of the malignancy (Malmgren, Bennison and McKinley, 1952; Linder, 1962; Prehn, 1963). These and other observations have prompted several investigators to consider the impairment of the immune response as a necessary step in the pathogenesis of a malignancy, the basic idea being that the normal immune response effectively suppresses most malignant growth (Thomas, 1959; Prehn, 1964). The various experimental studies of lymphocytic malignancies emphasize the primary role of the primordial lymphoid tissue in the development of lymphoma as well as the obverse relationship, i.e., the effect of the lymphoma on the immune response.

Clinical Studies

Immunologic Deficiency Diseases

A number of distinct immunologic deficiency syndromes have become apparent during the 12 years that have passed since Bruton (1952) described the first case of agammaglobulinemia (Good, Kelly, Rötstein and Varco, 1962; Barandun, 1959). These syndromes are distinguished by their clinical and pathologic features and in many circumstances by their familial occurrence. The frequent association of immunologic deficiency syndromes with thymic abnormality and lymphoreticular malignancies places studies of these patients in the center of any inquiry into the clinical association of such phenomena. The general clinical classification of the several rather distinct types of agammaglobulinemia can now be supplemented with data suggesting the pathogenesis of the disorders thereby enabling a more systematic approach to their study and treatment (Peterson, Cooper and Good, 1965).

Swiss Type of Agammaglobulinemia

This severe form of agammaglobulinemia is named in recognition of the investigators in Switzerland who discovered and defined the syndrome (Barandun, 1959; Glanzmann and Riniker, 1950; Hitzig, Biró, Bosch and Huser, 1958; Tobler and Cottier, 1958; Cottier, 1964). Children with this problem have been seen all over the world (Allibone, Goldie and Marmion, 1964; Donohue, 1953; Rosen, Gitlin and Janeway, 1962; Gitlin and Craig, 1963; Sacrez, Willard, Beauvais and Korn, 1963; Good et al., 1963). Clinical attention is focused on these patients very early in life because of their markedly decreased resistance to infections of bacterial, viral, fungal and protozoan origin. These children lack plasma cells, are unable to produce any detectable immunoglobulins and,

in addition, are deficient in small lymphocytes and, probably as a consequence, cannot develop delayed hypersensitivity or reject a skin homograft. The pathogenesis of this syndrome is probably the failure of these patients to develop either component of their lymphoid system. In all cases studied to date the thymus has been a vestigial epithelial organ devoid of lymphocytes and Hassall's corpuscles, the tonsils devoid of lymphoid follicles, and the lamina propria of their gut has been completely devoid of lymphocytes. Probably as a consequence of the failure to develop any primordial lymphoid tissue these children have a completely undeveloped peripheral lymphoid tissue. These children have invariably died of overwhelming infections in the first year and a half of life, usually before they are 6 months old.

This type of agammaglobulinemia is familial. Both boys and girls have been affected. Consanguinity has been reported in a few families, but no parents have been known to have had an immunologic deficiency. We have studied three girls and four boys, all with apparently identical syndromes; however, the family pattern of the last patient, a boy, suggests that his disease may have been inherited as an X-linked recessive trait. His two brothers and three other close male relatives died of overwhelming infections and either pre- or postmortem studies revealed that these persons all were immunologically deficient or possessed characteristics of immunologic deficiency. Three other males in the family also died of overwhelming infections while still infants. No females have been affected in this family. Gitlin's series of patients with the Swiss type agammaglobulinemia contains only males, a further indication that a sex-linked pattern of inheritance may exist in certain families (Gitlin and Craig, 1963). At the present time, it appears likely that the syndrome of the Swiss type agammaglobulinemia is the result of more than one genotypic defect, but the nature of the immunologic deficiency is superficially the same for all types: a failure of normal development of the gut-epithelium associated primordial lymphoid tissue.

Ataxia-Telangiectasia

Ataxia-telangiectasia is a syndrome characterized by a progressive cerebellar ataxia, oculocutaneous telangiectasia, and frequent sinopulmonary infections (Louis-Bar, 1941; Boder and Sedgwick, 1963). Symptoms begin in early childhood and the children usually die before adolescence with severe neurologic disability and compromising pulmonary infections. In addition, eight of these patients are known to have died from various lymphoreticular malignancies including lymphosarcoma, Hodg-

kin's disease, reticulum cell sarcoma, and generalized reticuloendotheliosis (Boder and Sedgwick, 1963; Szanto, 1963; Peterson, Kelly and Good, 1964). An immunologic deficiency is detectable in most patients on careful study (Thieffry, Arthuis, Aicardi and Lyon, 1961; Young, Austen and Moser, 1964; Fireman, Boesman and Gitlin, 1964; Peterson, Kelly and Good, 1964). More than 50 per cent of the patients have low or absent γA globulin, and in a few patients a marked deficiency in γG globulin is also present (Peterson, Kelly and Good, 1964). Delayed hypersensitivity and the homograft rejection mechanism are markedly impaired. Six patients we have studied were defective in their ability to develop delayed allergy to 2-4-dinitrofluorobenzene, a stimulus which readily produces delayed hypersensitivity in nearly 100 per cent of immunologically normal persons, and were very deficient in their capacity to reject skin homografts. These children have a variable lymphoid system deficit as assayed by the appearance of their tissues. Most of them have poorly developed lymphocytic collars around the germinal centers of their lymphoid follicles. A few are also deficient in plasma cells. Little attention had been paid to the primordial lymphoid structures of these children until recently when such structures became important in our thoughts concerning the immune system. Out of approximately 100 known cases the thymus was noted to be absent in four cases at postmortem. We have since biopsied the thymus from two of our patients and obtained one more autopsy specimen. A very characteristic thymic abnormality existed in all three of these patients.

The thymuses were composed almost completely of epithelial-stromal elements; no Hassall's corpuscles and only very few lymphocytes were present. Apparently the thymus was arrested at the epithelial stage of development and never differentiated to the lymphoid organ. Mesenchyme is necessary for the induction of an epithelial thymus to a lymphoid thymus (Auerbach, 1960) and since a mesenchymal defect, reflected in the generalized vascular abnormality (the widespread telangiectases), is the predominant feature of this disease, it is possible that such a mesenchymal defect is also responsible for the failure of thymic induction and consequently for the failure of the immune system to develop fully. The immunoglobulin abnormality and the occasional absence of plasma cells in these patients may reflect the interdependence of the two components of the immune system, or may indicate a basic defect in both systems.

The frequent association of this syndrome with various lymphoreticular malignancies may provide a clue to the pathogenesis of such malignancies. The experimental models of lymphocytic malignancies in mice and

chickens strongly support the contention first voiced by Kaplan et al. (Carnes and Kaplan, 1956; Kaplan, 1960) that only relatively undifferentiated cells are susceptible to oncogenic agents. In the mouse such cells are present in the newborn thymus; in the chicken such cells are located in the bursa of Fabricius (Peterson and Good, 1965). The incompletely developed thymus present in patients with ataxia-telangiectasia may remain a site of relatively undifferentiated lymphocytes all during the patient's life and consequently an appropriate oncogenic agent will find a cell population especially susceptible to malignant transformation. If such is the case, these patients exhibit the association of immunologic deficiency and lymphoreticular malignancy because of a common defect: the failure of normal thymic development.

The familial occurrence of ataxia-telangiectasia is well documented (Boder and Sedgwick, 1963). Boys and girls are affected with equal frequency and no parents have had symptoms of the disease. A few instances of consanguineous marriages are known to have produced children with ataxia-telangiectasia in keeping with the concept that this disease is transmitted as a simple autosomal recessive trait. In summary, we would propose that the genetic determinant in ataxia-telangiectasia results in production of a defective mesenchyme, and that the subsequent developmental failure of the thymus and the thymic-dependent peripheral lymphoid tissue is secondary to that defect. The abnormalities of the immunoglobulins may be secondary to the thymic defect or may indicate a concurrent defect in the development of the immunoglobulin-producing system.

Bruton's Congenital X-Linked Agammaglobulinemia

This severe form of agammaglobulinemia, first described by Bruton in 1952 (Bruton, 1952) is the prototype of all the immunologic deficiency syndromes. It is probably best to identify this disease with Bruton's name for it will help to distinguish this from the other types of agammaglobulinemia that may also be inherited as X-linked recessive traits. Children with Bruton's type agammaglobulinemia have a normal appearing thymus, and normal numbers of blood lymphocytes, but lack developed organized peripheral lymphoid organs, true lymphoid follicles, and plasma cells (Good, 1954a,b, 1955; Good, Kelly, Rötstein and Varco, 1962; Gitlin, Janeway, Apt and Craig, 1959). They cannot produce significant amounts of any circulating antibody, but most can manifest delayed hypersensitivity (Kulneff, Pedersen and Waldenström, 1955; Porter, 1957; Good, Bridges, Zak and Pappenheimer, 1959). Their homograft rejection mechanism is generally deficient in that such patients are

less able to reject a skin graft promptly than are normal individuals. These children have a gross deficiency of their pharyngeal lymphoid tissues (Margulis, Feinberg, Lester and Good, 1957), and this may be a clue to the pathogenesis of their condition. The lymphoid tissue of normal palatine tonsils is morphologically quite similar to that of the chicken's bursa. Bruton's type of agammaglobulinemia is probably the result of a failure to develop the immunoglobulin-producing system, and these children are in this respect comparable to the bursectomized chicken: able to develop delayed hypersensitivity but unable to produce circulating antibody.

The absence of plasma cells in these children has prompted study of the capacity of their lymphocytes to transform in tissue culture into the large blast-like cells considered by some to be plasma cells. Fudenberg and Hirschhorn (1964) demonstrated that the small lymphocytes from children with agammaglobulinemia could be transformed in vitro by phytohemagglutinin to the large cells they considered to be plasma cells. On the basis of this observation they postulated that the genetic defect in such children is not in the production of plasma cells but in the production of the gamma globulin molecule, a molecule that itself may normally induce the transformation to plasma cells. Although this hypothesis is attractive, it has not stood up to critical analysis. The major criticism is that the larger transformed cells are not plasma cells by the criteria usually accepted for the definition of such cells (Robbins, 1964). In addition, other investigators have not observed the transformation of small lymphocytes from patients with agammaglobulinemia (Elves, Roath and Israëls, 1964). A more theoretical objection to the hypothesis of Fudenberg and Hirschhorn is that in order for a person to be completely unable to produce any immunoglobulins he would have to have at least four and probably more gene defects because at least that many polypeptide chains are necessary for the construction of the γG, γA and γM antibody molecules. It seems much more economical of genetic information to postulate one defect concerned with the development of an entire cell line as the basis of the observed deficit. This latter hypothesis is supported by studies of experimental agammaglobulinemia in chickens deprived of their bursa-dependent cells.

In addition to their immunologic deficiency, approximately one-third of the children with this type of agammaglobulinemia have had rheumatoid arthritis of a character indistinguishable from that which occurs in immunologically intact persons (Janeway, Gitlin, Craig and Grice, 1956; Good, Rötstein and Mazzitello, 1957). Others of these patients have had dermatomyositis (Page, Hansen and Good, 1963; Cock, Rosen

and Banker, 1963), regional ileitis, thrombotic thrombocytopenic purpura, and other inflammatory vascular disorders. In addition to "autoimmune disease," three children with Bruton's type agammaglobulinemia have died of acute lymphatic leukemia (Page, Hansen and Good, 1963; Reisman, Mitani and Zuelzer, 1963). One of these latter patients also had dermatomyositis.

The hereditary pattern of this syndrome is almost certainly that of an X-linked recessive trait. Only boys have been involved in such families; parents and sisters have been clinically well. Serologic study of the normal family members has generally revealed no rheumatoid factors, antinuclear factors, or other immunoglobulin abnormalities (Fudenberg, German and Kunkel, 1962), although the mothers of two of our patients have elevated gamma globulin levels. The association of agammaglobulinemia, lymphocytic malignancies, and autoimmune diseases in this genetically determined syndrome supports the postulate of a common basic pathogenesis for all three phenomena.

Primary "Acquired" Agammaglobulinemia

The severe immunologic deficiency that occurs in patients with the "acquired" form of agammaglobulinemia is not congenital but develops usually after 30 years of age. Most of these patients have very low levels of all the immunoglobulins and cannot produce detectable antibodies in response to antigenic challenge (Sanford, Favour and Tribeman, 1954; Good, 1954a; Zinneman, Hall and Heller, 1954; Rosecan, Trobaugh and Danforth, 1955). Rheumatoid arthritis occurs frequently both in the patients with this form of agammaglobulinemia and in their family members (Fudenberg, German and Kunkel, 1962). Several have had a Coombs positive hemolytic anemia despite their general immunologic unresponsiveness (Fudenberg and Solomon, 1961; Good, 1954a; Prasad, Reiner and Watson, 1957; Traggis, Ruthig, Smith and Cleveland, 1961; Kunkel, 1964; Crockett, 1964). Thymomas have occurred in at least 13 patients (Pope and Osgood, 1953; Ross, Finch, Street and Streider, 1954; Good, 1954a; Ramos, 1956; Martin, Gordon and McCullough, 1956; Lambie, Burrows and Sommers, 1957; Gafni, Michaeli and Heller, 1960; Wollheim and Waldenström, 1962; Peterson, Cooper and Good, 1965; Godfrey, 1964). These thymomas are regularly benign spindle cell tumors and their removal does not alter the immunologic deficiency. Two patients with the adult type of agammaglobulinemia have later developed malignancies of their lymphoreticular tissues (Fudenberg and Solomon, 1961; Recant and Hartroft, 1962). Many others

have had large nodes and spleens secondary to hyperplasia of the reticulum cells. Although the etiology and the pathogenesis of this disease is unknown, the simultaneous occurrence in these patients of autoimmune diseases, thymoma and lymphoreticular malignancies, and the frequent occurrence of similar conditions in family members suggests that these patients may be keys to the understanding of several disease states. Several patients with a disease otherwise typical of so-called acquired agammaglobulinemia have had an associated disorder of the lymphoid tissue characteristic of benign follicular lymphoma.

The familial aspects of primary "acquired" agammaglobulinemia have only recently been clarified. Wollheim (Wollheim, Belfrage, Cöster and Lindholm, 1964) studying several families of such patients in Sweden found four cases of this syndrome in two kinships. Two were fifth cousins and two were third cousins once removed. Obviously, these relationships would not have been detected if it were not for the accurate family records maintained in Sweden. Furthermore, the family members of other patients with this syndrome have been found to have a high frequency of autoimmune diseases and serum protein abnormalities (Fudenberg, German and Kunkel, 1962). Hypergammaglobulinemia has occurred in several relatives, plasma cell myeloma in one, rheumatoid arthritis in many, and rheumatoid factor in approximately 25 per cent of the immediate family members. Further support of a familial abnormality is provided by a family studied by Wolf et al. (Wolf, Gokcen and Good, 1963). They reported a family in which the propositus had "acquired" agammaglobulinemia and rheumatoid arthritis, and close relatives had idiopathic thrombocytopenia, fatal lupus erythematosus, regional ileitis, rheumatoid arthritis, and complex abnormalities of the immunoglobulins. An uncle died of lupus and was found at postmortem to have a thymoma.

These family studies suggest that primary acquired agammaglobulinemia is not a sporadic, truly acquired disease, but rather one conditioned by an underlying genetic abnormality which is generally of late expression and may manifest itself in several ways. The normal stable state and function of the immune system indicates the existence of homeostatic control mechanisms which probably involve a negative feedback mechanism. Exact definition of a homeostatic system controlling lymphoreticular tissue is not possible with available knowledge, but the clinical and experimental observations that have been made outline the crude shape of a mechanism worthy of study. Patients with primary acquired agammaglobulinemia and various other abnormalities

of the lymphoreticular tissues probably reflect an abnormality in such a homeostatic mechanism and should continue to provide clues to the nature of the normal controls.

Aldrich's Syndrome

Aldrich's syndrome is characterized by eczema, dermatitis, thrombocytopenia, decreased resistance to infections of bacterial and viral origin, and familial occurrence in boys (Wiskott, 1937; Aldrich, Steinberg and Campbell, 1954; Lindberg and Palmgren, 1962). The exact basis of the very pronounced susceptibility to infections is unknown (Krivit and Good, 1959), although several abnormalities in the lymphoreticular tissues are apparent (Palmgren and Lindberg, 1963; Cooper et al., 1964). Many of these patients have low serum levels of the γM macroglobulins; all either fail to develop or develop very late the circulating isohemagglutinins against heterologous blood group antigens (Krivit and Good, 1959); and most are unable to manifest delayed allergy. Lymphopenia has been a fairly consistent finding, and the peripheral lymphoid tissues of these patients are deficient in lymphocytes, being made up primarily of reticulum cells. The thymuses from these patients appear normal.

At least three patients with Aldrich's syndrome are known to have died with tumors composed of reticulum cells (Kildeberg, 1961; Coleman, Leikin and Guin, 1961; ten Bensel, 1964) another example of the simultaneous occurrence of a genetically determined disturbance of the lymphoreticular tissues and a lymphoreticular malignancy.

The frequent familial occurrence of Aldrich's syndrome and its exclusive occurrence in males strongly suggests that it is inherited as an X-linked recessive trait. The extensive family tree published by Aldrich et al. (Aldrich, Steinberg and Campbell, 1954) is characteristic of inheritance by an X-linked recessive mechanism. Until more is known concerning the pathogenesis of the disturbance, however, the modus operandi of the genetic defect will remain undefined.

Autoimmune Diseases

The term autoimmune disease encompasses a great variety of clinical syndromes that often have in common only the presence in the patient's serum of an antibody that combines with an antigen obtained from some human tissue (Doniach and Roitt, 1962). Despite this loose definition of what must certainly be a complex assortment of heterogeneous diseases, there is a cohesiveness about several of these syndromes that makes the general designation useful. It may seem confusing to con-

sider these human disorders under the heading of lymphoreticular disorders, for already these autoimmune diseases have also been labeled as mesenchymal, connective tissue, and collagen disorders (Venters and Good, 1963); however, in the context of this discussion such a perspective is justifiable. Indeed, classifications are not so much right or wrong as they are useful or not useful. We will consider here just two of the many autoimmune diseases. These two, systemic lupus erythematosus and rheumatoid arthritis, were chosen because they are considered to be typical of autoimmune diseases, they have been extensively studied, and, in addition, at least in the case of lupus erythematosus, often have morphologic abnormalities in the thymus and other lymphoreticular tissue.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is the prototype for all the other autoimmune diseases. The signs and symptoms of multiple organ system involvement and the widespread vascular abnormalities are generally so consistent from one of these patients to another that the syndrome is clinically very distinctive (Harvey et al., 1954; Peterson, Vernier and Good, 1963). The serum of these patients often contains autoantibodies that react with almost every imaginable tissue of the body and, indeed, as a group the patients with SLE seem quite exempt from the general adage that an organism does not produce antibodies against "self" (Dameshek, 1958). Multiple cases of SLE have been reported in more than 40 families, and, although this disease is much more common in women than in men, both sexes have been involved in several families (Peterson and Good, 1963). The families of patients with SLE also contain a greater number of individuals with rheumatoid arthritis, rheumatoid factor, antinuclear factors, and other immunoglobulin abnormalities than would be anticipated to occur by chance alone (reviewed, Peterson and Good, 1963; Leonhardt, 1964).

Considerable clinical and pathological overlap exists between SLE and other autoimmune diseases. This is especially so with rheumatoid arthritis, dermatomyositis, and thrombotic thrombocytopenia (Peterson and Good, 1963). It has recently become evident that SLE and myasthenia gravis are also closely associated, often occurring simultaneously in the same patient (Hess, Eliasson, Grigson and Ziff, 1964; White and Marshall, 1962). This latter association is of particular interest for it is well known that patients with myasthenia sometimes have a thymoma and usually an abnormal thymus containing germinal follicles and many plasma cells (Castleman and Norris, 1949; Sloan, 1943). Such a

thymic abnormality is thought to be similar to that observed in the NZB mice previously discussed. Similar thymic abnormalities have also been observed in several patients with SLE without evidence of myasthenia gravis (Larsson, 1963; Mackay and deGail, 1963; Mackey, Masel and Burnet, 1964). Specifically what is occurring in the patients with SLE and in their families is unknown, but in light of the studies of experimental autoimmune diseases it seems reasonable to postulate that the pathogenesis of SLE involves an abnormality of the cellular basis of the immune system. Whether or not this perspective will be helpful in the future study of these patients and their families remains to be seen.

Rheumatoid Arthritis

Rheumatoid arthritis is generally considered as another of the autoimmune diseases because of the frequent presence in the serum of macroglobulin antibody directed against the patient's own gamma globulin. This antibody now extensively studied and defined is called the rheumatoid factor (Waaler, 1940; Rose, Ragan, Pearce and Lipman, 1948). Further, there exists clinical and pathological overlap with SLE, dermatomyositis and other of the diseases thought to represent autoimmune syndromes (Peterson and Good, 1963; Kunkel and Williams, 1964). In addition, rheumatoid arthritis has a clinical association of special interest in the context of this review, that is its frequent occurrence in patients with immunologic deficiency syndromes, specifically Bruton's type and the adult "acquired" form of agammaglobulinemia. Rheumatoid arthritis is also a familial disease occurring 2½ to 5 times as frequently in family members of persons with rheumatoid arthritis as in the general population (Holman, 1962; Peterson and Good, 1963).

The etiology of rheumatoid arthritis is as obscure as that of SLE (Venters and Good, 1963; Kunkel and Williams, 1964). The periods of high fever and leukocytosis observed in some patients coupled with the frequent occurrence of the disease in patients with an immunologic deficiency suggest that an infectious agent may underlie the entire process, but this relationship remains speculative. The pathogenesis of rheumatoid arthritis is likewise obscure, but there are some clues. A few patients with rheumatoid arthritis have had a thymoma (Burnet and Mackay, 1962; Lattes, 1962) and considering the association of rheumatoid arthritis with SLE, rheumatoid factor, and immunologic deficiency states the pathogenesis of this disease may also involve a basic defect in the immune system. Patients with Sjögren's syndrome (Sjögren, 1933; Bloch and Bunim, 1963), a syndrome usually comprising keratoconjunctivitis and xerostomia in addition to rheumatoid

arthritis have, in several instances, been reported to have a lymphoreticular malignancy also (Talal and Bunim, 1964). Other autoimmune diseases have also been associated with lymphoreticular and other malignancies (Williams, 1959; D. G. Miller, 1962; Page, Hansen and Good, 1963; Cammarata, Rodnan and Jensen, 1963). These associations weave even more tightly the intriguing triad of autoimmune disorders, immunologic deficiency states and lymphoreticular malignancies.

Lymphoreticular Malignancies

Lymphoreticular malignancies are among the most common neoplastic diseases of man. Lymphatic leukemia, lymphosarcoma, Hodgkin's disease and reticulum cell sarcoma are the generally recognized morphologic types, and although it is clinically helpful to distinguish them for purposes of treatment and prognosis, it is at the same time worth noting their close relationships. They all arise from the lymphoreticular tissues, and at times it is difficult to distinguish one from the other. The clinical association of lymphoreticular malignancies with immunologic deficiency syndromes and autoimmune diseases is a most provocative observation and probably an important clue to the pathogenesis both of these malignancies and perhaps of the "autoimmune disorders." It seems a reasonable working hypothesis that the same lymphoreticular cell defect that underlies the immunologic deficiencies predisposes to the malignancy of the lymphoreticular cells. The same relationship may pertain between lymphoreticular malignancies and certain autoimmune disorders. This as yet undefined, but real relationship is highlighted by a boy we recently studied who had agammaglobulinemia, dermatomyositis and a lymphoma (Page, Hansen and Good, 1963).

There are also other relationships of autoimmune disease, immunologic deficiency states and malignancy that merit mention. Not infrequently autoimmune phenomena such as LE cells and Coombs positive hemolytic anemia will occur in patients with a lymphoreticular malignancy (Rappaport and Johnson, 1955; Buffa and Rappaport, 1957; Brody and Finch, 1961). These phenomena probably reflect the aberrant activity of the disturbed immune system (Ebbe, Wittels and Dameshek, 1962). It is also not at all uncommon to have an immunologic deficiency follow the development of a lymphoma (Jim, 1957; Creyssel et al., 1958; Fairley and Scott, 1961).

There are familial aspects to the lymphoreticular malignancies as there are to immunologic deficiency states and autoimmune diseases that deserve emphasis. The literature contains numerous reports of lymphocytic malignancies in the same family (Anderson and Hermann,

1955; Hitzig and Rampini, 1959; Dameshek, Savitz and Arbor, 1929; Jelke, 1940; Videbaek, 1947; Riel, 1948; Cooke, 1953; Guasch, 1954; Kaliampetsos, 1954; Wegelius and Paaso, 1956; Johnson and Peters, 1957; Aleksandrowicz and Blicharski, 1960; Gross, 1961a). This has been especially true in identical twins where the odds are great that if one twin has the disease the other will also soon develop the same disease. Reticulosarcoma has also been reported in identical twins (Zachau-Christiansen and Rasmussen, 1963). Probably the most dramatic and tragic example of familial leukemia occurred in the family reported by Anderson (1951). Here five out of eight children died of lymphatic leukemia. The familial occurrence of leukemia by no means, of course, proves a genetic basis, especially in view of the very real possibility that this disease has an infectious origin. Studies such as that of Steinberg (1960) also clearly demonstrate that these lymphocytic malignancies do not occur with increased frequency in most families.

The new knowledge concerning the two specialized types of lymphoid tissue present at least in higher animals may offer some insight into the study of malignancies of this tissue. It is now known that most lymphocytic malignancies in the mouse involve the thymic-dependent lymphoid tissue and in the chicken the bursal-dependent tissue. Efforts to construct a classification of lymphoreticular malignancies based on this concept of two distinct types of lymphoid tissue in man seem attractive. For instance, Hodgkin's disease is characterized by an impairment of the mechanism for cellular hypersensitivity but an apparently intact immunoglobulin producing system. This combination directs attention to the thymic-dependent tissue as the primary site of involvement. On the other hand, in myeloma one may be seeing in bold relief evidence of involvement of the "bursal" type or productive component of the lymphoid tissue. Benign follicular lymphoma may also be another type of involvement of the follicular or bursal-type component.

The studies previously discussed suggest that several factors must be operative for a lymphoreticular malignancy to develop: an oncogenic agent, generally a virus; genetic susceptibility, probably related to cell receptor sites; and a population of relatively immature cells which can be induced to develop as malignant cells. The families that contain the multiple cases of lymphoreticular malignancies probably are characterized by the simultaneous occurrence within the members of the family of all three factors, at least two of which, the proper receptor sites and the susceptible cell population, may be genetically determined. The frequent lymphoreticular malignancies in the children with

ataxia-telangiectasia emphasize the hazard of retaining an immature thymic lymphocyte population all through life. Even though an endemic agent may account for some familial cases of leukemia, the simultaneous presence of an immature or in some other way defective lymphoreticular tissue is almost certainly necessary for the development of the malignancy.

Summary

The clinical and experimental evidence relating three ostensibly distinct disease states of man suggests a common basis for these conditions. Immunologic deficiency states, autoimmune diseases and lymphoreticular malignancies have many overlapping features: they often occur simultaneously in the same patient and the same experimental animal, and what is particularly provocative, all three entities occur with greater than anticipated frequency in certain families. A consideration of the pathogenesis of the clinical conditions and of the experimental models suggests that disturbances of the thymus and other lymphoreticular tissue may underlie all three phenomena.

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CHAPTER 2

The Genetics of the Glycogen Storage Diseases

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THE GLYCOGENOSES are of particular historical interest to the biochemically oriented geneticist because they were the first of the inborn errors of metabolism to have the enzymatic defect demonstrated definitively (Cori, 1953). The first clinical description of one of this group of conditions with marked hepatomegaly was by Parnas and Wagner in 1922 and later by van Creveld in 1928. The first necropsy report was by von Gierke in 1929. Pompe (1933) described a patient with glycogen accumulation with symptoms related most prominently to the heart. Cori (1953) delineated four distinct diseases of glycogen storage. The first was due to a deficiency of glucose-6-phosphatase in the liver and kidneys, the second to a generalized increase of normal glycogen in most tissues of the body, and two were characterized by the presence of structurally abnormal glycogen. Since then two further types have been defined enzymatically (see Table 1) (Mommaerts et al., 1959; Schmid, Robbins and Trant, 1959; Hers, 1959).

The delineation of the enzymatic defect in the various types of glycogen storage disease has not only permitted a clearer understanding of this group of diseases but it has also clarified our understanding of the metabolism of glycogen. It is also obvious that an accurate and unequivocal definition of the disease is essential before it is possible to think in terms of assessing genetic transmission, calculating the prevalence of the disease, or devising tests for heterozygosity.

Some knowledge of the metabolism of glycogen and the more im-

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TABLE 1.—*Classification of Glycogen Storage Diseases*

Type	Defect	Structure of Glycogen	Involvement
I	Glucose-6-phosphatase	Normal	Liver-kidney
II	Acid α 1,4 glucosidase	Normal	Generalized
III	Amylo-1,6 glucosidase and/or oligo-1,4 → 1,4 glucotransferase	Limit dextrin	Generalized, liver, muscle
IV	amylo-1,4 → 1,6-transglucosylase (not proved)	Amylopectin like	Generalized
V	Muscle phosphorylase	Normal	Muscle
VI	Liver phosphorylase	Normal	Liver

mediate interrelations is required to appreciate the various types of glycogen storage disease and the possible approaches to testing for heterozygosity. Some reactions of glycogen metabolism and certain in vivo interrelationships which are not yet clear have a bearing on our understanding of the glycogenoses. The metabolism of glycogen has been well reviewed (Hers, 1964a; Ciba Symp., 1964); the following brief summary emphasizes recent advances.

STRUCTURE OF GLYCOGEN

Glycogen is composed entirely of glucose units attached in series, chiefly in α 1,4 linkage; that is, one glucose is attached at its number 4 carbon to the number 1 carbon of the next glucose unit. This obviously would result in a straight chain molecule, but it has been shown that glycogen is a branched chain molecule. The branches have been shown to result from glucose linked through its 6 carbon to the number 1 carbon of the next glucose unit. It is estimated that there are three glucose residues between branch points in the structure and that the terminal branches have 7 to 12 glucose units (French, 1964).

The physical characteristics of extracted glycogen vary markedly with the method of extraction; it appears that cold water extraction yields a glycogen similar to that present in vivo. Using this method, muscle glycogen has a molecular weight of 6 million, and liver glycogen from 60 to several hundred million. Liver glycogen is markedly polydisperse, with the percentage in the heavy weight fraction varying with the nutritional state of the animal. Electron microscopic study reveals that glycogen exists in the cytoplasm of the cell as discrete spheres which may occur singly, as units of several spheres, or as large aggregates. Molecular weight estimation by electron micros-

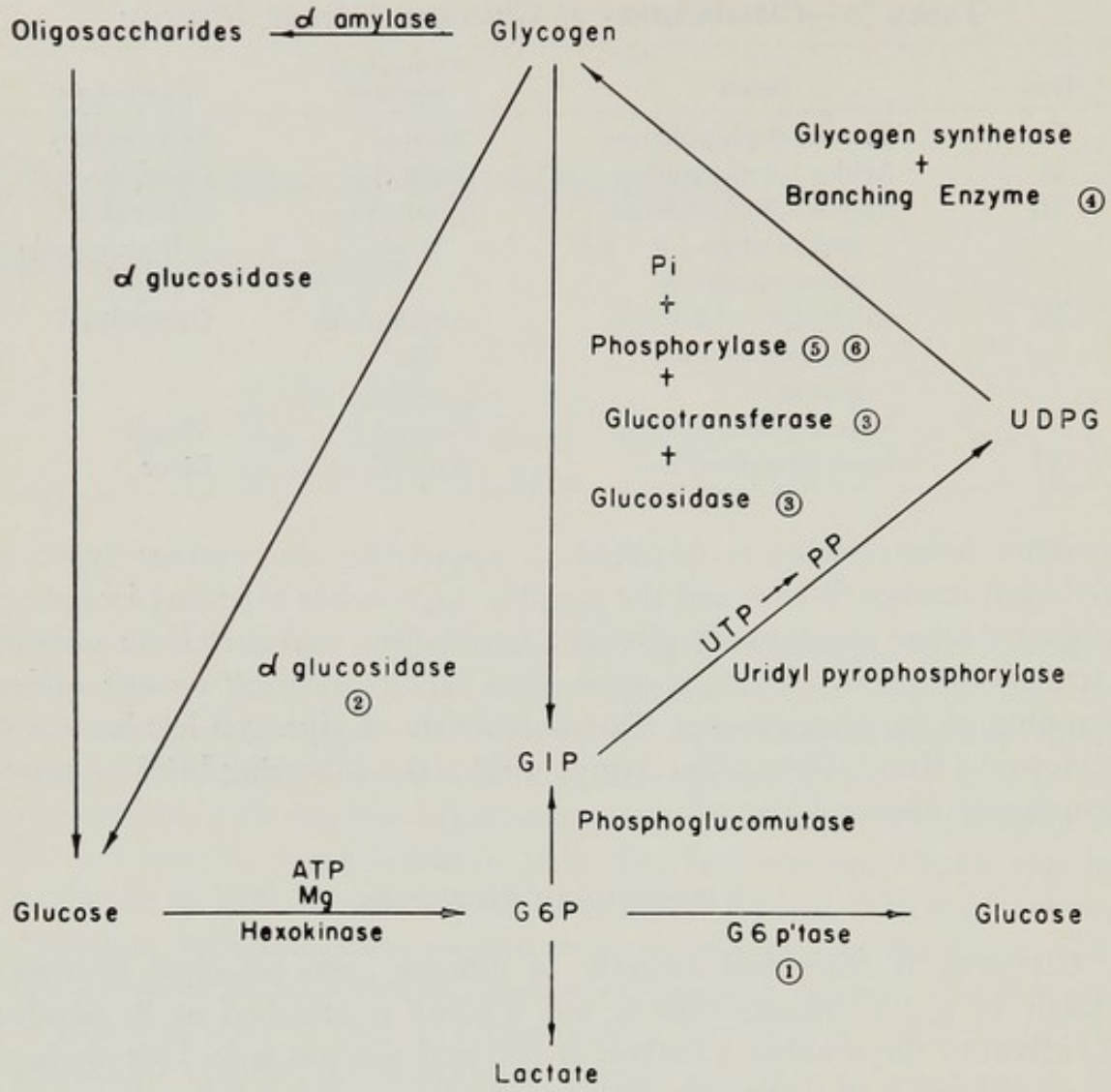

FIG. 1—Schema of glycogen metabolism. The enzymes deficient in the specific types of glycogen storage disease are indicated by the corresponding number beside the enzyme. The α-glucosidase indicated to be associated with type II glycogenosis is the lysosomal glucosidase, that shown associated with α-amylase is the neutral, nonparticulate α-glucosidase.

copy confirms the large average molecular weight shown for cold water extracted glycogen (Orrell, Bueding and Reissig, 1964).

THE ENZMYES INVOLVED IN GLYCOGEN SYNTHESIS

Synthesis of the α 1,4 linkage by the enzymatic reaction utilizing uridine diphosphoglucose (UDPG) (Fig. 1) was first shown by Leloir (1957). It would appear that this is the primary pathway for the α 1,4 glycosyl addition and the enzyme appears to be part of the endoplasmic reticulum. The branch points, or α 1,6 linkages have been shown to result from the action of the enzyme amylo-1,4 $\rightarrow$ 1,6 trans-

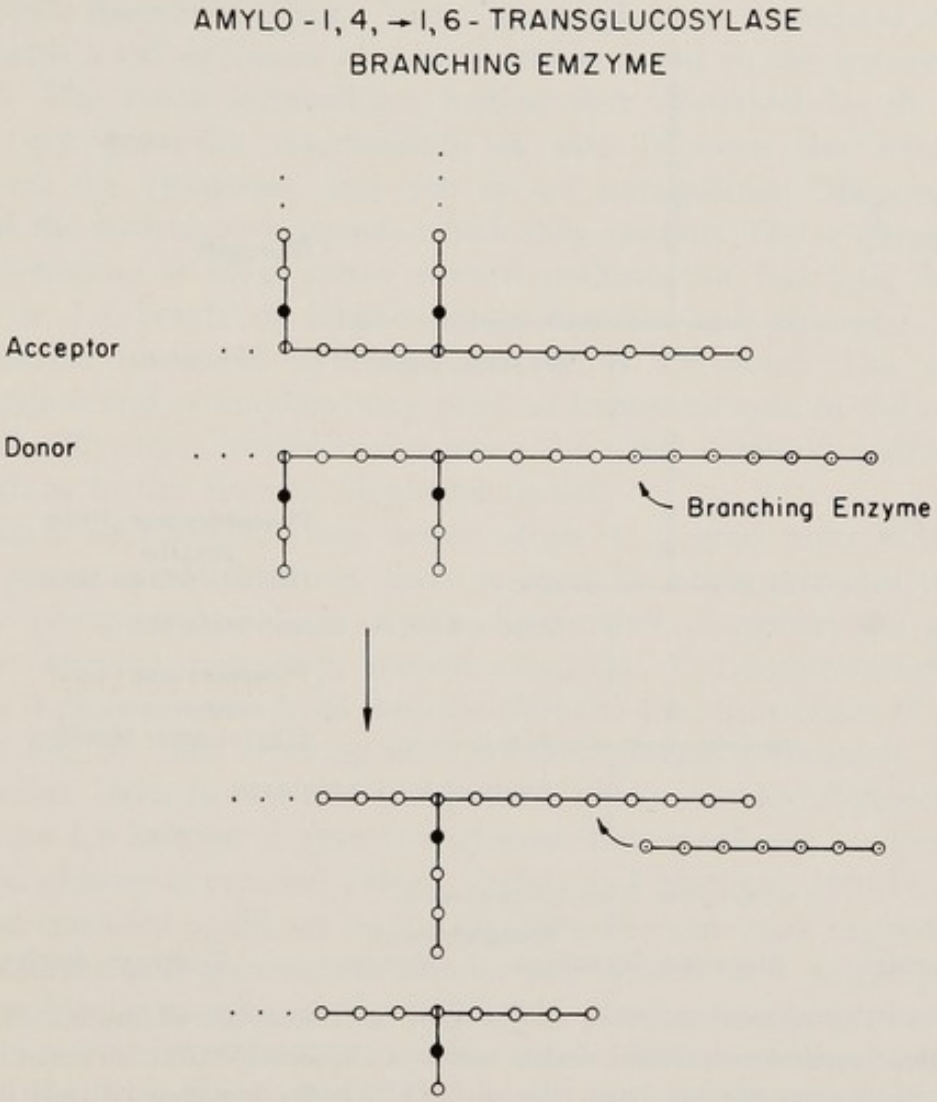

FIG. 2—The proposed mechanism of the branching enzyme is illustrated (after French, 1964).

glycosylase (French, 1964). This enzyme acts as an oligotransferase, in that when the terminal glucose residues exceed eight or more in number, this enzyme transfers four or more glucose residues to the stem structure by a 1,6 linkage and thereby generates a branch point (Fig. 2). Thus the branch-structured glycogen is formed.

THE ENZYMES INVOLVED IN GLYCOGEN DEGRADATION

The phosphorylase reaction (Fig. 1) was shown to be freely reversible under proper laboratory conditions and was held for a time to be responsible for both synthesis and degradation of glycogen. It is generally agreed now that the in vivo action of phosphorylase on glycogen is catabolic. Phosphorylase in the presence of inorganic phosphate

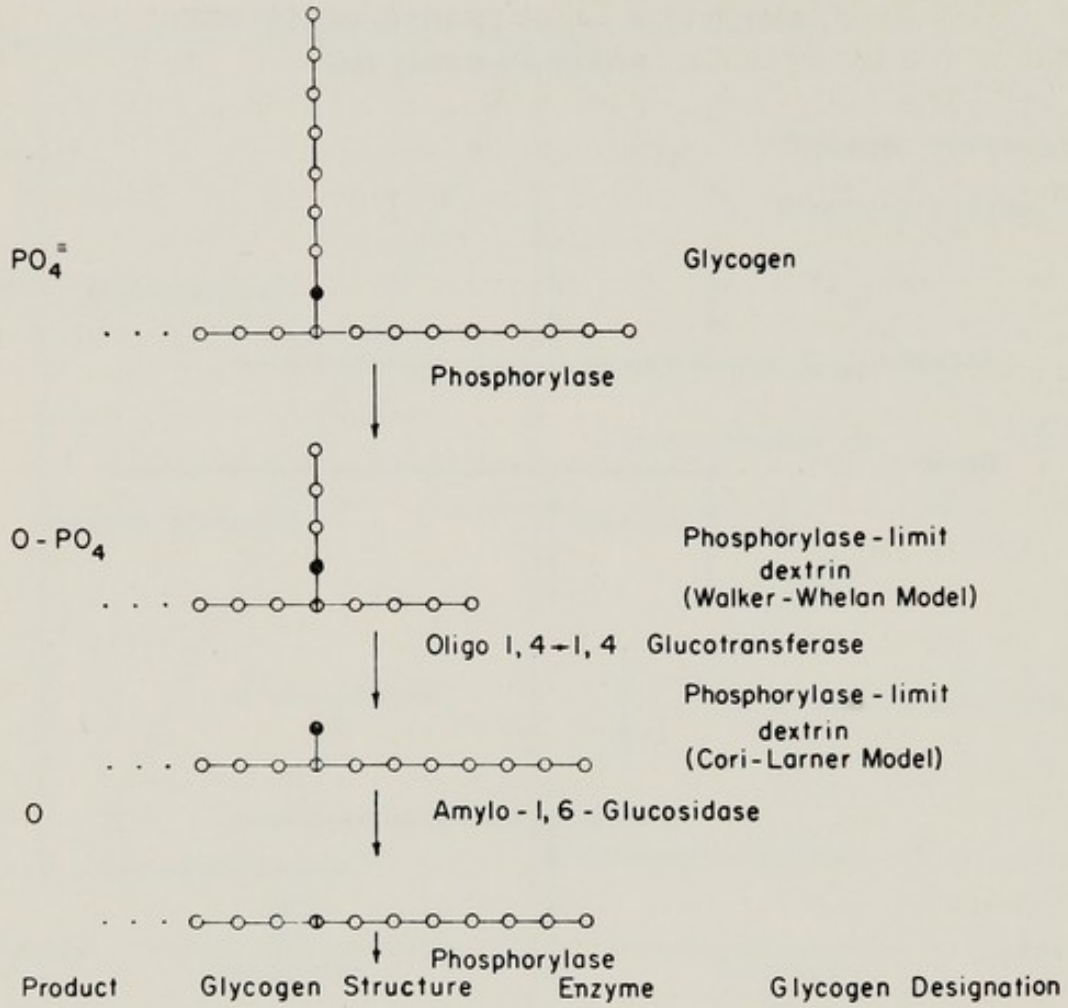

FIG. 3—The coupled reactions of glycogen catabolism are illustrated. Phosphorylase action generates a limit dextrin with a Walker-Whelan structure and the oligo-1,→4-glucotransferase bares the α-1, 6 linkage branch-point which is then substrate for amylo-1, 6-glucosidase action. Once the branch-point is removed, phosphorylase can again proceed.

will remove one glycosyl residue at a time from the terminal branches of glycogen until 4 glucose units from the branch point (the α 1,6 linkage) remain (Fig. 3). Phosphorylase is nonparticulate in the cell. Active phosphorylase, phosphorylase a, is a dimer of inactive phosphorylase, phosphorylase b. The phosphorylase cycle, relating to polymerization of of phosphorylase b and depolymerizing phosphorylase a (Krebs et al., 1964), is important in the activity measurements of phosphorylase and in time it is highly probable that a glycogen storage disease will be defined in relation to this system. There is a second pathway for degradation of glycogen, which has come to the fore only recently, involving α glucosidase (Fig. 1). It has been shown that there are at least two α glucosidases in the cell which have different pH optima, substrate specificities, and intracellular localizations (Lejeune

et al., 1963; Rosenfeld, 1964). The α glucosidase of immediate interest is that with a pH optimum of 4 or 5 and is found in the lysosomes of the cell. The latter intracellular bodies, first described by de Duve (1955), are generally intermediate in size between the mitochondrion and the ribosome, and act as an intracellular "disposal" by virtue of the hydrolytic enzymes which they contain. The α glucosidase of the lysosome is most active toward maltase but has lytic activity for the α 1,4 bonds of large oligosaccharides and glycogen. There is no known lysosomal hydrolase for the α 1,6 bond. The neutral α glucosidase and α amylase may play an important role in the regulation of the glycogen concentration when the usual catabolic pathway is impaired, as in the absence of phosphorylase.

Inasmuch as phosphorylase action stops 4 glucose units from the branch point, as noted above, there is need for other enzymes to prepare the phosphorylase limit dextrin for further phosphorylase action. The first enzyme necessary, named oligo-1,4 → 1,4-glucotransferase, transfers 3 glucose units from the side chain to the main chain of glycogen (see Fig. 3), thus exposing the 1,6-linked glucose (Abdullah, Taylor and Whelan, 1964; Brown and Illingworth, 1964). Amylo-1,6-glucosidase cleaves the 1,6 linkage to give free glucose but it will not act until the 1,6 linked glucose is exposed (Hers, Verhne and Mathieu, 1964d). Phosphorylase can then again act on the glycogen. Recent work suggests that the transferase and glucosidase activity are located on a single protein with a molecular weight of about 270,000. Various methods were employed in an attempt to separate the two activities without success. Hence it would appear to be a bifunctional enzyme (Brown and Illingworth, 1964).

The glucose-1-phosphate formed by the action of phosphorylase in the presence of inorganic phosphate may be converted to glucose-6-phosphate by phosphoglucomutase. In the liver and kidney glucose-6-phosphate has several possible fates, one of which is hydrolysis to glucose, catalyzed by glucose-6-phosphatase (Fig. 1). This reaction in liver is basic to the regulation of the blood sugar level.

Clinical Picture

The classification of the glycogenoses in Table 1 reflects the historical sequence of enzymatic definition or clinical recognition and is based on that originally proposed by Cori (1953). Some have preferred a classification on the basis of the major organ system involved, i.e., the hepatomegalic form would include types I, III, IV, and VI; and the muscular form would include types II and V. The latter has virtue when consider-

ing differential diagnosis, but limitations are becoming apparent in the light of recent developments, and one must ultimately refer to the former classification which described the enzymatic defect involved.

Types I, III and VI, generally speaking, are clinically indistinguishable. The clinical manifestations depend on the age at which the individual is studied. In the neonatal period those with type I are more likely to be symptomatic. They may show hepatomegaly, respiratory distress, hypoglycemia, convulsions, and ketonuria. This is one of the few conditions in which ketonuria is found in the neonatal period. Strangely, prolonged jaundice and hemorrhagic tendencies are rarely noted (Gabilan, 1960). The condition may not be detected until later in the first year during routine examination. In those individuals with symptoms, frequent bouts of diarrhea with steatorrhea are reported (Crawford, 1946). Intermittent episodes of unexplained pyrexia occur. Muscular weakness is the rule. These children generally are slow to stand and walk but not to talk. The IQ is usually normal.

The liver usually attains tremendous size toward the end of the first year of life, which is also the time when the serum triglycerides and cholesterol increase markedly. Xanthomata have been noted only in types I and III. It is toward the end of the first year also that these individuals begin to show a poor response to infection. Minor viral infections which have little effect on other members of the family may be associated with hyperpyrexia, ketosis, acidosis, a toxic state, and a relatively prolonged course. Death can ensue if active measures are not taken to treat the acidosis. A low fasting blood sugar is the rule, indeed, in some individuals the blood sugar will not be measurable in the morning after an overnight fast, yet they will be alert and attentive. Hypoglycemic convulsions are found primarily in type I glycogenosis, but they are also seen in type III. At about 12 to 18 months the individual begins to have bleeding episodes, associated usually with infections. The bleeding is usually from the nose and may at times be severe enough to lower the hematocrit significantly. Postoperative bleeding from extraction of teeth, and removal of tonsils and adenoids has been a significant problem, whereas bleeding has not been a problem with liver biopsy as a rule. Petechiae or ecchymoses are uncommonly noted but a prolonged bleeding time is the rule. Several patients in our experience have been thoroughly investigated for impaired circulating clotting factors with negative results. This finding is considered to be the effect of altered metabolism on the arterioles and veins resulting in delayed or ineffective constriction after injury. Abnormal platelet function has also been suggested (Gabilan, 1960). By 1 year

of age there is obvious growth retardation, but the child appears fat and round-faced. The growth retardation persists but the inadequate handling of infection improves gradually over several years, and the fasting blood sugar level increases. Rarely hypoglycemic convulsions may persist for several years. The triglyceridemia does not usually return to normal in patients with type I glycogenosis. The liver tends to diminish somewhat in size with age but remains grossly enlarged. Puberty is usually delayed. It is becoming evident that the late effects of type I glycogenosis relate to the secondary findings, particularly the markedly elevated serum uric acid. Uric acid stones and gout are frequent among those individuals studied (Jeune, Charrat and Bertrand, 1957; Jeandet, 1961). Two patients have died at about 40 years of age with gouty nephritis (Holling, 1963). Early coronary artery disease can be anticipated with persistence of marked hyperlipemia.

We have seen all of the childhood symptoms of type I in type III glycogenosis except xanthomata. The child with type III may have the same dwarfed, cherub appearance which is so common in type I; however, the milder cases of type III do not have this appearance. It is often stated in the literature that severe ketosis and bleeding diathesis are not seen in type III glycogenosis; our most troublesome bleeder has been a patient with type III glycogenosis and his fasting ketosis has equalled any type I patient of his age. The prognosis for type III would appear to be quite different from that of type I. The symptoms and findings, including the size of the liver, decrease significantly around puberty. Usually by puberty these children are ostensibly normal. The triglyceridemia has returned to near normal although the cholesterol may remain somewhat elevated. The uric acid is not markedly elevated, but frequently above normal. The two patients originally reported by van Creveld in 1928 and 1932 have recently been shown to be of the type III variety. Both are of normal size and healthy, aged 44 and 40 (van Creveld and Huijing, 1963). One has one child, the other two; all are normal. Oliner, on the other hand, has seen one man with type III glycogenosis who at about 40 developed a myopathy; he has two apparently affected children (Larner, 1964). We have seen one man and one woman who developed a myopathy around the age of 40. Another man, age 30 years, is in ostensibly good health, but has a grossly abnormal EKG. At this point one must hold in abeyance the answer to the question regarding the long range prognosis of patients with type III glycogenosis.

Very few clinical reports of type VI have been published (Lamy et al., 1960; Gabilan, 1960). Our experience is in agreement with the re-

ported cases. These patients rarely have symptoms other than growth retardation during the first year. The liver enlargement may not be detected until the second year of life. None have shown the bleeding tendency so prominent in type I and sometimes seen in type III. The obesity and cherub-like appearance of the face has not been noted consistently in type VI; such patients may be normal or even gaunt. The uric acid level is significantly elevated in those patients in whom it has been tested (Lamy et al., 1960; Sidbury, unpublished observations). The serum lipids are also elevated. One cannot at this time make any statement about the long-range prognosis. There has been only one patient reported beyond puberty (Gabilan, 1960). There is no uniformity of enzymatic definition of type VI.

The two patients reported with type IV glycogenosis were noted at about 1 year of age to have an enlarged liver and spleen (Andersen, 1956; Sidbury et al., 1962). The liver was hard and nodular and the spleen firm. In one instance the liver function tests were grossly abnormal, in the other they were unimpressive. Growth was normal until approximately the first year of life. The course was that of any cirrhosis of the liver. Hypersplenism and repeated hematemesis were seen. The cases reported by Craig and Uzman (1958) probably represent examples of type IV glycogenosis. They demonstrated an increase in mucopolysaccharide in the liver of one of their patients but did not characterize the glycogen.

The two forms of glycogenosis which affect primarily the muscle are in fact different one from the other. Type V glycogenosis was originally described by McArdle (1951) in a man who had a long history of cramps and stiffness and who became exhausted easily with minimal exercise. Schmid and Hammaker (1961) have studied a family with three patients having type V glycogenosis. They describe the following clinical phases: (1) childhood and adolescence when fatigability is the only symptom; (2) the period from 20 to 40 years when severe cramps on exertion associated with occasional transient myoglobulinuria are prominent; and (3) the period after 40 years when individual muscle groups become increasingly weak and wasted.

Type II glycogenosis is classified with the group of glycogenoses affecting muscle because the symptoms relate primarily to muscle. The symptoms in these patients may begin at birth with feeding difficulties, dyspnea, and cyanosis. On the other hand, the infant may apparently thrive for 2 or 3 months and then begin to develop symptoms, and lose those motor skills which he had attained. The picture is one of progressive weakness, dyspnea with exertion, loss of reflexes, and pharyngeal

pooling in the final stages, with aspiration and difficult swallowing. In the final stages there is a virtual flaccid paralysis. In contrast to the marked weakness, the muscles are full, firm, even hard. The heart size increases progressively over the several months and becomes very large, due to biventricular enlargement. The EKG becomes progressively abnormal; the outstanding features are the shortened P-R interval, the very large QRS complex, and the inversion of the T waves. Arrhythmias are frequent, especially the gallop rhythm. Death usually follows a respiratory infection. Cardiac decompensation is frequently seen.

Accessory Findings and Enzymatic Lesion

(1) In type I glycogenosis there is an absence of glucose-6-phosphatase (Fig. 1), an enzyme found normally only in the liver, kidney and intestine (Cori, 1953; Ockerman, 1964; Field, Epstein and Egan, in press). The enzyme is absent functionally and by direct assay at all ages in the patients studied. Certain glycolytic intermediates have been shown to be elevated in the liver (Brante, Kayer and Ockerman, 1964). The liver glycogen content generally ranges between 6 and 12 per cent. The very low blood sugar found in infants with this condition, in contrast to older children and adults, suggests the greater importance of this pathway for the regulation of blood sugar in the very young. There is a significant elevation of the fasting blood lactate at all ages. Because of the positive arteriovenous difference for lactate it is deduced that the increased lactate derives from glycogen breakdown in the liver which cannot be converted to glucose (Mason, 1943). Similarly when glucagon or epinepherine is administered there is a significant rise in lactate but not in glucose in the blood. When galactose is given intravenously there is a prompt rise in blood glucose in the normal which is not seen when glucose-6-phosphatase is absent (Schwartz, 1957). Usually about the end of the first year of life there is a significant and persistent rise of the serum lipids. There is an elevation of blood uric acid which arises presumably by the same mechanism as the hyperuricemia associated with starvation, but is probably accentuated by the increased tubular reabsorption secondary to elevated blood lactate (Howell, 1962; Reem, 1964). Finally there have been several determinations which indicate that the serum hydrocortisone level is elevated, whereas the urine concentration is low (Jeune et al., 1959). We have confirmed this in three patients and demonstrated a prolonged half-life of serum hydrocortisone in one. This would suggest the possibility of a failure of the feedback or homeostatic mechanism

TABLE 2.—*Enzymatic Defects in Type III Glycogenosis (Hers 1964c)*

	Amylo-1,6-Glucosidase		Oligo-1,4 → 1,4-Glucotransferase	
Subtype	Liver	Muscle	Liver	Muscle
A	Deficient	Deficient	Deficient	Deficient
B	Deficient	Present	Deficient	Deficient
C	Present	Deficient	Present	Present
D	Present	Present	Deficient	Deficient

regulating the level of the blood hydrocortisone level and possibly an impediment in the dehydrogenation of hydrocortisone by the liver. We have demonstrated normal glucuronidation of salicylate in these patients.

(2) The defect in type II glycogenosis is an absence of α 1,4 glucosidase (acid maltase) which has been shown to be a lysosomal enzyme (Hers, 1963, 1964, a,b; Lejeune, 1963).

Hers (in press) has recently extended his original observation of an absence of α glucosidase, by showing with electron micrographs that the lysosomes of the liver of these patients are engorged with glycogen, whereas the extralysosomal glycogen is normal. This demonstration explains a number of findings in these patients, e.g., the normal carbohydrate metabolism (see Table 2), the presence of glycogen accumulation in the presence of normal amounts of active phosphorylase and amylo-1,6-glucosidase, and the progressive course of the disease. The glycogen within the lysosome is insulated from the phosphorylase which is nonparticulate, and located in the cytoplasm. Electronmicroscopy of the liver of patients with other types of glycogen storage disease shows an entirely different distribution of the glycogen in the cell (Salmon, Habib and Bernhard, 1961; Sheldon, Silverberg and Kerner, 1962). The liver glycogen content ranges from 5 to 10 per cent, the heart from 3 to 8 per cent, and striated muscle from 6 to 15 per cent. This distribution of accumulated glycogen is of interest for several reasons. The distribution of glycogen does not reflect the quantitative distribution of lysosomes so far as is known, indeed the liver is considered to be more richly endowed with lysosomes than either heart or striated muscle. Recent work suggests lysosomes in the infant heart are reduced relative to the adult, but a proper stimulus can cause them to develop. Further, the level of glycogen accumulated gives strong support to the thesis that this route of glycogen degradation is physiologically significant. Nonetheless it is not at all clear to this author why or how an absence of lysosomal α 1,4 glucosidase results in a diseased state, in particular one in which the patients manifest these symptoms.

It is possible that the improvement in the fasting blood glucose that is seen in other types of glycogen storage disease may reflect the in-

crease in lysosomes that occur with age. Similarly the increase in the neutral glucosidase may play an important role; nothing is known of the change in neutral glucosidase with age.

(3) The early studies of patients with type III glycogenosis suggested that like type II, it was a generalized disease, i.e., involving muscles and organs. Recent work by Hers has extended the original observations and complicated the picture. The various combinations of deficiencies resulting in a limit dextrin type glycogen are shown in Table 2. The glycogen in these patients has short outer branches; hence, there is very little substrate for phosphorylase to act on. Thus when the liver is involved the blood sugar tends to be somewhat lower than normal but only infrequently are there hypoglycemic convulsions. There is no marked hyperlactic acidemia as in type I, for obvious reasons. There is a variable degree of hyperlipemia which parallels the severity of the disease. The red cell glycogen is usually elevated and the glycogen present has the characteristic abnormality (Sidbury, 1961a). It is not clear why the patient becomes asymptomatic about the time of puberty. The decrease in liver size parallels the decrease in lipemia. This observation shows that the notable degree of hepatomegaly in glycogenosis is due more to the storage of fat than of glycogen, for liver biopsies on the adult with type III glycogenosis still shows a large concentration of glycogen, comparable to that found in the prepubertal patient.

(4) The glycogen formed in type IV glycogenosis also is abnormal, but in this instance the structure resembles amylopectin of corn starch, having fewer branch points than normal glycogen. Although not conclusively proven, the implication is that the amylo 1,4 → 1,6 transglucosylase is deficient, though clearly not absent. It is worth emphasizing that the concentration of glycogen is not elevated although its structure is abnormal. It is also difficult to extract from the tissue. It is assumed that this abnormal glycogen, being far less soluble in water than normal glycogen, precipitates and acts as a foreign body, thus giving rise to the cirrhosis of the liver which entirely dominates the clinical picture (Andersen, 1956). There is a possibility that the decreased amylo-1,4 → 1,6 transglucosylase activity is secondary, and the inhibition of activity results from the accumulated products of another deficient or absent enzyme. The generalized distribution of the abnormal glycogen would make this less likely. Except for the finding of an abnormal glycogen in the red cells (Sidbury, 1961a) there are no observations in these patients which lead one to suspect an abnormal glycogenosis. The clinical laboratory findings are those of any cirrhosis with hepatocellular damage and portal obstruction.

(5) The glycogen in type V glycogen storage disease is normal as

determined by enzymatic degradation (Roland, 1963; Sidbury, unpublished) and by sedimentation characteristics (Bueding, 1964). The enzymatic lesion is a complete absence of muscle phosphorylase (Mommaerts, 1959; Schmid, 1959); the liver phosphorylase being normal. These observations show the separate genetic control of liver and muscle phosphorylase. The various clinical laboratory methods of assessing glycogen and glucose metabolism show no abnormality except the lactate response to anoxic exercise. Because of the absence of phosphorylase in the muscle these patients show no rise of blood lactate consequent to anaerobic exercise (McArdle, 1951). It is not clear, however, why these individuals should show weakness on exertion, or be subject to muscle breakdown with severe exercise with consequent myoglobinuria. It is of interest that the glycogen concentration in the muscle of these patients is of the order of 1 to 5 per cent, whereas that in type II is 6 to 15 per cent. This would suggest that the level of glycogen in the muscle of patients with McArdle's syndrome is lower by virtue of an intact α glucosidase system.

(6) Type VI glycogenosis is associated with a deficiency in liver phosphorylase; the muscle phosphorylase being normal (Hers, 1959). The symptoms and findings are similar to those of patients with type I and type III lesions. The blood lactate, lipids and uric acid are not so markedly elevated as in type I. The moderate elevation of red cell glycogen (Sidbury, 1961b) and depressed leucocyte phosphorylase level (Williams, 1961; Hulzman, 1961) may be of significant diagnostic assistance. The blood glucose response to glucagon and epinephrine is variable; there is no lacticacidemia resulting from their administration. Symptomatic hypoglycemia has not been described. Enzymatic degradation of the isolated glycogen reveals no abnormalities, but curiously the sedimentation pattern of this glycogen has a characteristic distribution (Bueding, 1964). Hers (personal communication) has repeatedly expressed puzzlement over how a 30 per cent reduction of liver phosphorylase can result in glycogen storage. The level of glycogen in the liver of these patients usually ranges between that found in type I and type III glycogenosis.

Genetic Data

As with many inherited conditions in man, the data are insufficient to make secure genetic conclusions. This difficulty is particularly evident with the glycogenoses. The precise enzymatic defect may be subject to change as the biochemical techniques for investigating them become

more sophisticated. This diminishes the usefulness of some of the cases reported earlier because, by current standards, adequate enzymatic evaluation was not carried out. A decade from now reviewers on the same subject are likely to make the same comment about currently reported studies. Attempts to detect the heterozygous state are dependent on a precise knowledge of and method for assaying the enzyme activity which is altered.

For the present, one must seek evidence of genetic transmission based on the sex of affected individuals, the distribution of cases within families, and evidence of increased frequency of consanguineous marriages in the families of affected individuals. It is to be hoped that in the future ways will be devised to demonstrate the heterozygous state.

Type I. The information which is useful from the genetic standpoint is scant for this type of glycogen storage disease. It may be said unequivocally that the disease is found with the same frequency in males and females. It may also be stated that thus far all parents have been normal. Further, there have been three matings involving an individual with type I glycogenosis known to us, and in each instance the offspring has not shown evidence of glycogen storage disease.

The most useful information reported on the frequency in families is that of Illingworth (1964b). The data reported and our own are tabulated in Table 3. There is no readily apparent explanation of the somewhat greater incidence of disease in these families over that expected from an autosomal recessive hypothesis. In both groups, there is the possibility of incomplete ascertainment of unaffected sibs. In our group, there were 5 patients without sibs. There were two instances of consanguineous marriage, and there was one instance of an affected cousin. Gabilan (1960) reviewed 50 reported cases which had been enzymatically proved; finding no cases among cousins and only one instance of consanguinity. The first patient described by Walker, Ziai and Bowman, (1961), though not enzymatically documented, would appear to be an example of type I glycogenosis. The parents were Iranian and first cousins. Holling (1963) has reported further studies on the first family related by Ellis and Payne (1936) and he has demonstrated the glucose-6-phosphatase deficiency in two of the three affected children. Three children of a maternal cousin have also been born and all appear to have the same disorder.

In summary, although the data are still inconclusive it appears that the disease is genetically transmitted as an autosomal recessive condition.

Various methods have been used to obtain evidence of decreased glucose-6-phosphatase activity in the liver in the parents of those affected,

TABLE 3.—*Genetics of Type I*

Source	Number of Families (probands)	Sibs	Not Affected	Expected	Affected	Expected
Illingworth (1964b)	23	43	27	32	16	11
Sidbury	25	48	32	36	16	12
Total	48	91	59	68	32	23

TABLE 4.—*Type II Glycogenosis—Family Data*

Source	Number Families	Number Children	Number Affected	Per Cent	Number Males	Per Cent	Number Females	Per Cent	Consanguineous Marriages	Per Cent
Present author	21	84	32	38.0	20	63.0	12	37.0	1	5.0
Literature	59	164	84	51.0	37	60.0	25	40.0	7	12.0
Total	80	248	116	47.0	57	61.0	37	39.0	8	10.0

but without success (e.g., epinephrine and glucagon tests) (Brante, 1964; Sidbury, unpublished observations). Liver biopsy has not been performed. Field (1965) has assayed the intestinal glucose-6-phosphatase in the parents of two patients and believes he can distinguish the heterozygote. The level of glucose-6-phosphatase is low in the normal intestine and controls, other than those applied for liver homogenate, had to be applied to demonstrate activity. It may be that the pH optimum for intestinal glucose-6-phosphatase is much broader than for liver and kidney.

Type II. In type II glycogenosis tabulation of cases is more satisfactory than for types I, III, and VI. The clinical course is distinctive and the histochemical findings of the muscle unique (di Sant'Agnese, 1950; Zelleweger, 1955). Now a specific enzymatic defect can be added (Hers, 1963). The first line of Table 4 summarizes our experience, the second tabulates cases from the literature, and the third line combines the two. It is apparent that the frequency of affected infants closely approximates a 50:50 distribution both in the material collected from the literature as well as in our series. When the probands are subtracted for the purpose of estimation of the percentage affected, the resulting figure is 21.4 per cent, which approximates the 25 per cent expected. In our material the affected child was an only child in one instance. In the reported patients this was found in 15 families. Two patients were illegitimate. The slight but definite preponderance of affected males over females is significant. There is a significantly higher frequency of consanguineous marriages in the reported cases than found in our series. This could be the effect of the weighting of reported cases from Europe and the middle Eastern countries where the incidence of consanguinity is higher than in the United States. In both groups consanguinity is significantly higher than in the random population (Morton, 1961).

In one of our families a maternal aunt had three infants who died before 1 year of age, and although suspected these were not confirmed cases. Similarly Gitzelman (1957) reported a cousin of one of his patients who died shortly after birth with heart failure. We had one instance of an affected male with a normal male twin. No data were available to determine the zygosity of the twins. There was a similar report of an affected male with a normal twin; the sex was not stated (Humphreys and Kato, 1934). There have been several reported instances of matings by a second marriage, where an affected infant had resulted from the first mating (Sprague, 1931; Friedman and Ashe, 1958; Stoeckle, 1961). None of the offspring of the second marriages have been affected with glycogen storage disease. In two, however,

one of the half-sibs had congenital heart disease. In one of our families a paternal cousin had congenital heart disease. In this family there was another paternal cousin with the Werdnig-Hoffman syndrome. Childs (1952) also reported a first cousin of an infant with type II glycogenosis who had the Werdnig-Hoffman syndrome.

Among the other associated anomalies there was one reported with pyloric stenosis (Muggia, 1936) and another, born of an incestuous union, had a hare lip, cleft palate, club foot, and a hydrocoele (Monnet, 1960). The most frequently reported associated anomaly is endocardial fibroelastosis. This occurred in 2 of 28 affected individuals in our series. It occurred in 4 of the 80 cases in the tabulation of Table 3. In addition Kelly and Andersen (1956) reported this association and Merhezi (personal communication) has such a case.

In an attempt to obtain an estimate of the prevalence of the condition and the gene frequency we have tried to determine the number of patients born with type II glycogenosis in North Carolina from 1950 to 1963. North Carolina has a relatively stable population. The pathologists and pediatricians of the state were consulted and the records of the State Health Department Division of Vital Statistics were reviewed. There were 1,560,679 live births during this period and four cases were detected. This would give a frequency of approximately 1 in 400,000 live births or about 1 heterozygote in 300 persons.

Type III. The situation with type III glycogenosis, while being clarified from the enzymatic point of view by Illingworth (1964a) and Hers (1964c), has been hopelessly confused for the moment from the vantage point of a geneticist. Hers (1964c) has detected only one example for each of two of the variants.

Gabilan (1960) reviewed 10 cases; in three of the families consanguinity was noted, which is extraordinarily high. Recently Illingworth (1964) reported 24 patients, 12 of whom had affected sibs. In the families with more than one affected, there were 27 children, 14 of whom were affected. It is the experience of all that the parents are normal and there is a significant number of siblings affected. We have four patients with type III glycogenosis who are married and have a total of six normal children. Oliner's (Larner, 1964) patient, an adult, had two children with evidence of involvement. The disease is found with equal frequency in males and females.

Table 2 tabulates the various combinations of deficiencies of amylo-1,6-glucosidase and oligo-1,4 → 1,4-glucotransferase which Hers (1964c) has encountered in his studies of liver and muscle biopsy material from patients with type III glycogenosis. On the basis of testing the glucosidase

and transferase he is able to define four subgroups. It is noteworthy that in the several combinations he has encountered, there are none in which the liver is deficient in both glucosidase and transferase and the muscle normal for both activities, or vice versa. There are examples of discordant deficiencies, vis-a-vis the two activities, both in muscle and liver. The biophysical data (Brown and Illingworth, 1964) indicate that these activities are associated with a single bifunctional enzyme, i.e., a single protein with two distinct enzymatic functions. The biochemical genetic data show that one or both of the activities of this enzyme may be altered and that the alteration of the activities in liver and muscle may be independent. It is difficult to conceive of these situations based on primary structural gene alterations. Similarly, it would be equally difficult to explain the findings by means of a control gene mutation. At the present it would appear that each subtype will ultimately have to be explained with different genetic mechanisms.

It is obvious that in the present uncertainty, it is premature to discuss the recognition of the heterozygous state. Preliminary efforts in this direction have been made in individual families. We have some evidence that the level of red cell glycogen may be a useful marker in some families. Hers (1964a) had similar data. We have not characterized the glycogen from these presumed heterozygotes, hence at this time this marker must be considered nonspecific. The studies of Williams (1963) and Steinitz (1962) suggest that the enzyme levels in leukocytes and erythrocytes will be useful for detecting the heterozygote. This approach deserves more extensive trial, measuring both the glucosidase and transferase activity.

The tendency for sibs to be affected, the equal occurrence in males and females and the high incidence of consanguineous marriages support the supposition that type III glycogenosis is an autosomal recessive condition. Recent developments in the biochemistry of the glycogen debranching mechanism and the application of this knowledge to patient material necessitates a delay in deciding whether all subtypes can be genetically classified.

Type IV. There are only two families reported with this type glycogenosis. In the first family there was a child who died of a similar illness (Andersen, 1955). In the second family, the patient was the only child of a second marriage for both parents, both having normal children by the first marriage (Sidbury et al., 1962). If, as suspected, Craig's (1958) cases represent the same condition, it is noteworthy that his first two patients were sibs. The two proved cases and the similar affected sib were all males, but Craig had two males and one fe-

male. Thus it is not yet clear whether the condition will be inherited in an X-linked or autosomal fashion. The parents in all instances have been normal. It would appear that none of these patients live more than several years. It seems likely that the disease is not nearly as rare as the scant data would suggest. Because the glycogen content is not excessive, the diagnosis is not usually considered. All biopsies of the liver in children showing gross scarring should have a glycogen analysis as well as enzymatic study. Only in this way will we in time be able to gain some idea of its prevalence, and the frequency of this type glycogenosis as a cause of liver cirrhosis in children.

Type V. Nine affected individuals have been reported in five families with a total of 23 children (McArdle, 1951; Schmid, 1961; Pearson, 1961; Roland, 1963; Mellick, 1962). Assuming single ascertainment, $P_e = .22$. There were two consanguineous marriages and an affected cousin in one family. All the affected individuals were males except one whose presumptive diagnosis was made post mortem by history. We have studied one affected male who had 4 normal sibs. The paternal grandfather had symptoms suggestive of the disease. The parents were not known to be related but both were born in a relatively isolated community. Mayer (personal communication) has studied members of two families with type V glycogenosis. In one family two brothers have been proved to have the condition and the third has identical symptoms but has not been biopsied. In the second family the younger boy has been proved to have the condition, an older brother has similar symptoms but refuses biopsy. They have an unaffected sister. Thus there are eight families with 34 children known to be at risk (excluding two males who died at 3 months of age), and 14 were affected; there was consanguinity in two of the eight families. Most of the families are of English or Scotch-Irish origin but one was from Colombia, South America and another immigrated from the Azores.

One family has been described with delayed onset of symptoms which imitate those found in McArdle's syndrome (Engel, 1963). There were eight sibs, two of whom were studied, and another had similar symptoms. The 52 year old female had no demonstrable muscle phosphorylase or rise in blood lactate in response to anoxic exercise. The older brother had a markedly diminished phosphorylase level but a normal rise of blood lactate in response to anoxic exercise. In both the muscle glycogen was within the normal range (1.1 per cent). This condition would appear to be different from McArdle's syndrome.

In type V glycogenosis there is a tendency for sibs to be affected, the parents are unaffected, and a high frequency of consanguineous

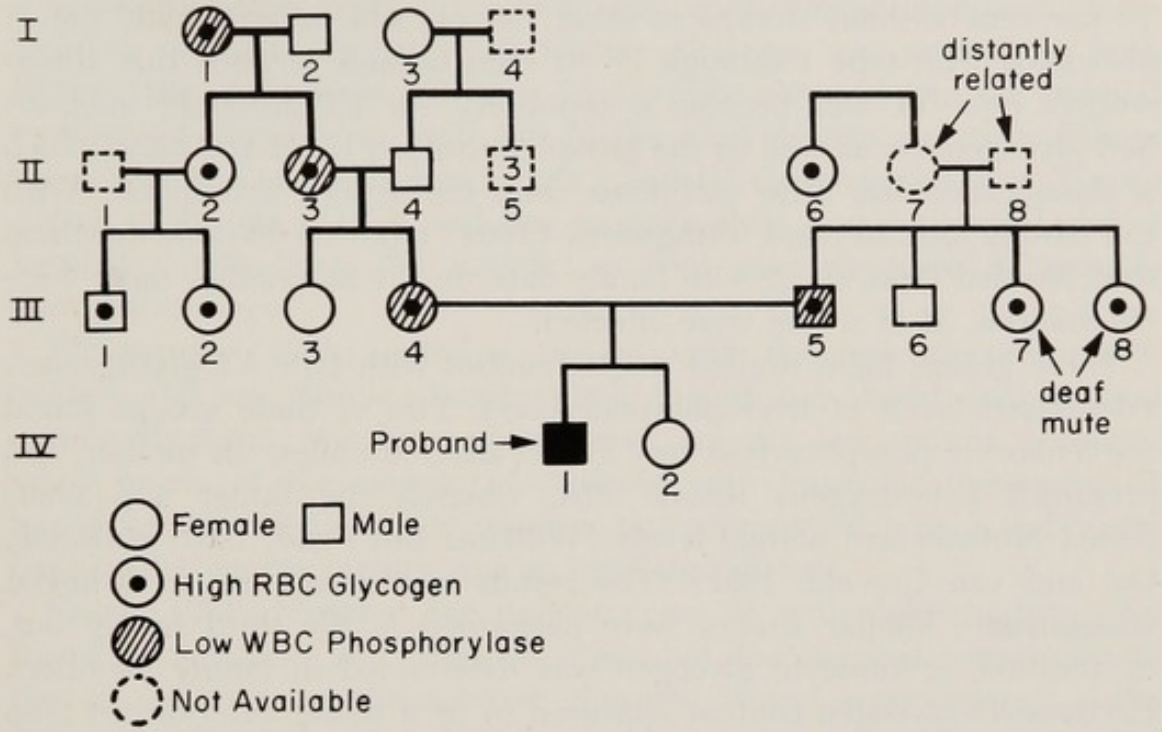

FIG. 4—The pedigree of a family with a proven case of type VI glycogenosis. Family members were studied by quantitating the red cell glycogen and the leucocyte phosphorylase in an effort to detect heterozygosity. The results are compatible with an autosomal recessive mode of transmission.

marriages is observed. One female is suspected of having the condition, but the diagnosis was retrospective. All proved cases have been in males which suggests that the condition may be X-linked or X-limited. The extensive genealogy reported in two families favors the latter (Schmid and Hammaker, 1961; Rowland, 1963). Note should be made of the unusually high ratio of males to females in these females, 28M, 8F; three had no sibs. Attempts to detect the heterozygote with the lactate response to anoxic exercise have not been successful.

Type VI. If there is confusion in the enzymatic definition of any of the glycogenoses, none can compare with that in type VI glycogenosis. Originally the classification was created to accommodate patients with a clinical picture resembling type I, with elevated liver glycogen, and liver phosphorylase the only enzyme with values at variance with the normal (Hers, 1959). The value of the liver phosphorylase was one-third to one-fourth of normal. Illingworth (1964), on the other hand, estimates that one-third of the material she studies from glycogen storage disease patients is categorized as type VI, which, however, is not necessarily a liver phosphorylase deficiency. She defines type VI as having the following characteristics: hepatomegaly, liver glycogen in excess of

12 per cent without storage in other tissues, and no enzymatic defect associated with type I through IV. It soon became obvious that Illingworth's type VI will become a repository for all presently unclassified glycogenoses as well as the group considered by Hers (1959). Such a classification has little usefulness from the genetic standpoint. With the criteria used in mind, Illingworth (1964) reported 36 patients whom she classified type VI, 25 with family data. In the 25 families there were 65 children, 32 of whom were affected.

Three groups have studied single families with type VI glycogenosis (depressed levels of liver phosphorylase). Two of these groups found the leukocyte phosphorylase very low in affected males, the mother, and presumed heterozygous female sibs, whereas the father and unaffected brothers had normal levels (Williams and Field, 1961; Hulsman. Oei and van Creveld, 1961). The results would support an X-linked transmission. Similar studies were performed in the third family but, in addition, erythrocyte glycogen was determined in family members. Erythrocyte glycogen content appeared to be a better determinant than the leukocyte phosphorylase value, but using either, the results were compatible with an autosomal recessive type of transmission (Wallis, Sidbury and Harris, in press). Females with this type of glycogenosis are reported (Lamy, 1960), but all our cases have been males.

Thus the genetic data relating to type VI glycogenosis is scanty. Three family studies have been undertaken, utilizing the level of phosphorylase in the leukocyte in two instances and the erythrocyte glycogen level as an additional parameter in the third family. In two families the condition appeared to be X-linked, in the third family the data were compatible with autosomal recessive transmission. The only report of the condition in a female comes from France (enzymatic assay by Hers). The very real possibility exists that two different types of transmission ultimately will be shown to exist in this type of glycogen storage disease.

Mixed and Dual Types. There have been several patients reported who would appear unequivocally to have more than one enzyme deficient. Similarly families have been reported with two sibs having different types of enzyme deficiencies.

Calderbank et al. (1960) have reported a pair of sibs, one of whom had type I, the other a type III glycogenosis. Perkoff (1962) and Steinitz (1961) have reported a patient with combined type I and III defects. Eberlein (1962) reported two sibs of interest. The younger had a type III defect in the liver and muscle, and a low glucose-6-phosphatase level in the liver. The older child had a pathologically low glu-

cose-6-phosphatase level in the liver, but, unfortunately, the liver glycogen was not characterized. The muscle glycogen was normal. In both siblings the erythrocyte glycogen was grossly elevated and the isolated erythrocyte glycogen in each was of the limit dextrin structure. Hence the older child probably had a type I and III defect in the liver, but no defect of muscle (IIIC) and the younger child had a type III defect involving both liver and muscle, as well as a depressed glucose-6-phosphatase level.

Illingworth (1961) noted a patient with deficient glucose-6-phosphatase and low muscle phosphorylase, though not absent, with normal muscle glycogen. We have encountered equally low muscle phosphorylase levels in the muscle of patients with muscular dystrophy. It would be difficult to decide whether this represented type I and V defects or the muscle picture was an unrelated disease.

Sokal (1961) reported one patient (case 2) with deficient liver glucose-6-phosphatase and phosphorylase, but the marked serum lactate rise in response to administered glucagon would argue against a deficiency of liver phosphorylase.

The interpretation of double defects must be approached with caution. There is great danger of creating double defects if the tissue is not properly handled at the time of biopsy and thereafter. Phosphorylase is the most labile of the enzymes usually studied. Further it has been our experience, and that of others, that frequently livers from patients with type III glycogenosis will show depressed levels of glucose-6-phosphatase and phosphorylase (Hers, personal communication).

It is tempting, following the work of Jacob and Monod (1961) in E. coli in which they demonstrated defects in enzymatic activity due to mutation of the regulator gene rather than the structural gene, to explain such double defects as a control gene defect. This approach is complicated by the diversity of the specific defects which are coupled. On the other hand, the probability of a patient having two recessive conditions in a closely related enzyme system is very small, and the number of reported double defects relative to the total number reported is high. The possibility exists that the combined type I and III defects are in fact type III defects with secondary suppression of glucose-6-phosphatase, through a deadaptive phenomenon. This would then leave the sibs reported by Calderbank and Manners (1960) to be explained, for each had a different defect. One would have to assume that both parents were heterozygous for both type I and III defects and that the two genes segregated.

Despite the increased clarification of the glycogenoses in recent years it is to be expected that patients with a clinical picture of a glycogen storage disease and an increased glycogen level in the tissues will be found, in whom no specific enzymatic defect can yet be demonstrated.

Summary

The clinical manifestations and associated findings, as well as the specific enzymatic defects of the different types of glycogen storage disease, have been interpreted within the framework of recent developments of the biochemistry of glycogen.

The scarcity of data on the specific types of glycogenosis which is useful for genetic analysis is noted. The information that is available suggests that types I, II, and III glycogen storage diseases are autosomal recessive conditions. The newer methods of assay have made it possible to subdivide type III glycogenosis into at least four subgroups. Although on superficial evaluation type IV glycogen storage disease appears to be a recessive trait, only two families have been reported. All affected patients have been males, but the number is too small to assume that this sex distribution is significant. All individuals with enzymatically proved McArdle's disease (type V) have been males, but one female sib had similar symptoms. It is suggested that this condition is recessive and X-limited; X-linkage cannot be excluded. The diagnostic criteria for type VI glycogenosis is the least satisfactory. Three families have been studied and in two the condition would appear to be X-linked, in the third family the data support autosomal recessive transmission.

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Chapter 3

The Inheritance of Common Congenital Malformations

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The malformations discussed in this article are selected from those common in Northwest Europe and North America. Among such malformations pride of place both for incidence and severity is taken by the two major central nervous system malformations dependent on failure of the neural tube to close, anencephaly and spina bifida cystica (myelomeningocele and meningocele). Second come the serious congenital malformations of the heart, if these are grouped together. Third comes mongolism (trisomy G_{21} or trisomy 21 syndrome). Probably no other severe malformations have an incidence of at least one in a thousand, though the incidence of trisomy E_{17} syndrome probably comes close to one in a thousand. In addition several less serious malformations—harelip (with or without cleft palate), talipes equinovarus, pyloric stenosis, inguinal hernia, congenital dislocation of the hip—have incidences of more than one in a thousand. It may be argued whether the latter three conditions are true congenital malformations in the sense of errors of development present at birth, but it is conventional and, in the author's opinion, justifiable, to include them. In indirect inguinal hernia the sac is almost certainly congenital. In infantile pyloric stenosis the anomaly is probably a true hyperplasia and has been seen in a child dying at the age of 3 days. Congenital dislocation of the hip may be fully present in stillbirths.

In this discussion the trisomy 21 syndrome is omitted as it was the subject of a recent review in this series (Lejeune, 1964) as also are the congenital heart malformations and indirect inguinal hernia as they have not yet been sufficiently studied.

Only in the case of the trisomy 21 syndrome does it appear that the

malformation is fully determined at zygote formation (or soon after zygote formation, if some instances of trisomy 21 originate from nondisjunction at an early mitotic cell division). With all the other common malformations there is evidence, from twin studies, that the etiology must include both genetic and intrauterine environmental factors, and also evidence, from family studies, that the genetic predisposition is multifactorial rather than due to a single major mutant gene; there is no evidence that chromosomal abnormalities play any part in their causation.

Twin Studies

The classic method of making a first estimate of the importance of genetic factors in the determination of any condition, by large scale studies of the cotwins of index patients, has not yet been fully exploited for common congenital malformations. For such studies large unselected series are needed and it is unsatisfactory to use individual case reports because of the bias which enters into the reporting of such pairs. The two best studies available are those by Idelberger for talipes equinovarus (1939) and congenital dislocation of the hip (1951). In both of these an attempt was made to ascertain all cases in a population. The type of twinning was determined by physical resemblance and not blood groups, but experience has shown that full blood grouping reveals few errors where classification by physical resemblance has been carefully made.

With congenital dislocation of the hip Idelberger had 29 monozygotic pairs, of whom 10, all female, were both affected. In a further two instances the cotwin of the index patient had marked acetabular dysplasia but no dislocation. The best estimate from this of the proportion of affected monozygotic cotwins of index patients will depend on the number of pairs in which both affected twins were independently ascertained index patients. If, in each of the concordant pairs, both were index patients (and this appears to be the case), the proportion of cotwins of index patients affected would be 20 in 39, or 51.3 per cent. If only one in each pair was an index patient the proportion would be 34.5 per cent. In contrast, none of 52 like sex dizygotic twins were concordant, while 3 of 57 unlike sex dizygotic twins were concordant. The over-all number of affected dizygotic twins of index patients was 6 in 112, that is, 5.4 per cent, since all affected were index patients. This percentage is similar to that being found in nontwin siblings of index patients. This twin series shows the major part genetic factors play in the causation of congenital dislocation of the hip, but also shows that environmental factors are important.

TABLE 1—*Concordance and Discordance in Published Twin Series of Pyloric Stenosis*

(C = concordant, D = discordant, M = male, F = female)

	Monozygotic				Dizygotic Like Sex				Dizygotic Unlike Sex		
	C		D		C		D		C	D	
	M	F	M	F	MM	FF	MM	FF	*MF*	*MF*	*FM*
Sheldon (1938)	–	–	1	–	–	–	1	–	–	–	–
Robertson (1940)	2	–	1	–	–	–	3	1	–	4	1
Cockayne and Penrose (1943)	–	–	–	–	–	–	–	–	–	2	1
Powell and Carter (1951)	–	–	2	1	–	–	1	–	–	–	–
Metrakos (1953)	1	–	–	1	–	–	–	–	–	1	–
MacMahon and McKeown (1955)	–	–	–	–	–	–	–	–	2	21	3

In the case of talipes equinovarus, Idelberger had 35 monozygotic pairs of whom eight (22.9 per cent) were concordant. Idelberger indicates that in five pairs both were index patients giving a concordance of 32.5 per cent. In contrast, only 3 of 133 dizygotic pairs were concordant. Idelberger indicates that in one pair both were index patients giving a concordance of 3.0 per cent. Genetic factors must play a major part in the causation of talipes equinovarus.

Idelberger and Idelberger (1940) also had a series of patients with harelip and cleft palate, but they were only able to visit a minority of the twin pairs ascertained in this study. Taking only twin pairs with the deformity of the type of harelip (± cleft palate), one of the two monozygotic pairs were concordant, one of the nine like sex dizygotic pairs and 2 of the 14 dizygotic pairs of unlike sex were concordant. In Fogh-Andersen's (1943) large Danish series, one of two of the monozygotic pairs were concordant, none of the eight like sex dizygotic and 1 of the 17 pairs of unlike sex were concordant. Summing these two unselected series, the proportion of monozygotic cotwins affected (assuming all are index patients) is four in six; but taking into account the single reports the true proportion affected is probably lower than this and less than 50 per cent. The proportion of dizygotic cotwins affected (assuming all are index patients) is 5 in 51.

Only small scale unselected twin studies are available for infantile pyloric stenosis and in some the type of twinning is only certain in the pairs of mixed sex. The findings in the main reports, where these are from unselected series, are summarized in Table 1 (references cited by Metrakos, 1953).

These are all hospital rather than population series and so in most pairs probably only one twin should be regarded as the index patient. The

proportion of male monozygotic cotwins also affected is 43 per cent, though this must be taken as only a very rough guide to the true proportion. No useful estimate may be given of the proportion of dizygotic cotwins also affected, but it is very probably less than that of the monozygotic cotwins.

Despite the high incidence of the malformation no large scale twin studies are available for anencephalus and spina bifida cystica; the affected twins are often dead before they have been compared with their cotwin to determine the type of twinning. Record and McKeown (1951) showed from hospital records that of a series of 16 like sex pairs (excluding those of unknown sex or fate) none were recorded as both affected, though five of the cotwins died in the perinatal period. Of six unlike sex pairs none were concordant. This series and individual case reports such as the discordant pair of Dumoulin and Gordon (1959) and the discordant monoamniotic pairs of Litt and Strauss (1935) and of Pedlow (1961) indicate that only a minority of monozygotic cotwins of index patients are also affected. There is no clear indication that this proportion is any higher than that of dizygotic twins also affected.

In the same series of hospital records, Record and McKeown (1951) found 17 index patients with spina bifida cystica who had a like sex cotwin and none of these were also affected, although four died perinatally; while of eight unlike sex pairs again none were concordant, though two died perinatally. However, their hydrocephalus patients included a stillborn like-sex twin pair both affected and these may well have had unrecorded spina bifida cystica, since the majority of cases of congenital hydrocephalus are secondary to myelomeningocele.

Twin studies, therefore, indicate that all these common malformations must be in part environmentally determined, but also give a strong indication, except perhaps in the case of anencephalus and spina bifida cystica, that genetic factors are also important. It is important to note, as mentioned in the discussion, that, for a condition with an incidence of one in a thousand, a monozygotic twin concordance of 40 per cent is compatible with a heritability of 80 per cent; but there are many reasons for caution in estimating heritability from data on twins.

Environmental Factors

Direct Evidence

There is no clear indication that environmental factors such as maternal infection by virus diseases, maternal ingestion of drugs or maternal therapeutic and diagnostic irradiation make an important contribution

to the incidence of the common congenital malformations with which we are concerned. Extensive retrospective history-taking from the mothers after the birth of such children has shown no consistent pattern of illness in pregnancy. Large scale prospective studies of events in pregnancy, apart from the already known influences—rubella infection, thalidomide and heavy dosage irradiation—show no association with congenital malformations, and rubella, thalidomide and irradiation do not produce any of the common malformations with which we are concerned. Intrauterine mechanical factors, notably those associated with the frank breech malposition, are important in the etiology of congenital dislocation of the hip and perhaps talipes equinovarus.

It seems probable that though there must be environmental factors among the causes of the common malformations, they are not of the type which are obviously noticeable during the mother's pregnancy.

There are, however, several indirect lines of evidence for the importance of environmental factors.

Social Class

One association which one might expect to find is between socio-economic class of the family, as judged by the husband's occupation, and congenital malformations. There is a very striking association of social class with infant mortality as a whole, and a definite, but rather less strong, association with perinatal mortality. There appears, however, to be only one association of social class with a common malformation and that is with anencephaly and possibly, to a lesser extent, with spina bifida cystica. This is shown most clearly in the data from Scotland where the causes of stillbirths have been registered since 1929 (Edwards, 1958; Anderson, Baird and Thomson, 1958). The incidence per thousand total births for anencephalus varied from 0.89 for the wives of men in professional and managerial occupations, to 3.60 for the wives of unskilled workers, a fourfold increase. For stillbirths and neonatal deaths from spina bifida cystica there was a similar but, less marked association, the range being from 0.17 to 0.31, rather less than a two-fold increase. A smaller apparent association of social class and deaths from hydrocephalus could be due to an admixture of cases of spina bifida with secondary hydrocephalus.

Undoubtedly there are numerous genetic differences between patients in different social classes; but, prima facie, such differences in incidence by social class suggest the importance of environmental factors, in the etiology of these major malformations of the central nervous system. Nutritional differences are one possibility, but, the areas men-

tioned in the next section, where incidence is high, are also areas where nutrition is good.

Regional Incidence

Common malformations, with the probable exception of the trisomy 21 syndrome, show striking variations in regional incidence (McKeown and Record, 1960). This has been especially well documented for anencephalus and spina bifida (Penrose, 1957). The highest recorded incidence of anencephaly has been reported from Ireland (Stevenson and Warnock, 1959), some areas of Scotland (Edwards, 1958), South Wales (Laurence, 1964), and Liverpool (Smithells, 1964), all areas in the north and west of the British Isles. These are also areas with a high incidence of spina bifida cystica, although the variation is less striking with this malformation. Such regional variations could be due to variation in genetic or environmental influences, and in general the two possible types have not yet been distinguished. Studies of the incidence in immigrant communities compared with the areas from which they emigrated will be especially valuable here. For example, the finding that Sikhs and Chinese have very different incidences of anencephaly in Singapore (Searle, 1959), make it improbable that these differences are due to climatic factors, but these two communities differ in many cultural habits, as both have largely retained the cultures of their native countries. In Australia the incidence of anencephaly and spina bifida is only about half that of Britain (Collman and Stoller, 1962) and this deserves further investigation to see to what extent the difference can be explained by the recent immigration from central and southern Europe, or by earlier immigration being mainly from southeast England.

Some of the exceptionally high incidences of congenital dislocation of the hip, for example, in Lapps (Mellbin, 1962) and some American Indians (Kraus and Schwartzmann, 1957) can perhaps be explained by a postnatal environmental factor, the tight swaddling of the limbs in a position of hip extension and adduction, which is likely to push certain cases of hip subluxation into dislocation, but a high incidence of congenital dislocation of the hip does not occur in all tribes that practice swaddling of their infants. A fall in the incidence of the malformation in Navajo Indians has plausibly been attributed to the introduction of diapers (Rabin et al., 1965).

Seasonal Incidence

A seasonal variation in incidence of congenital malformations has been reported for anencephaly, congenital dislocation of the hip, and

harelip. That for anencephaly was reported from Scotland and Birmingham, England by Record and McKeown (1951), who noted a relative incidence of about 1.3 for winter, as compared to summer births, and this was confirmed by Edwards (1958) on further Scottish data, in which the higher incidence in winter births was seen without exception in every year from 1939 to 1955. It is interesting, however, that no similar variation for anencephaly was found in Rhode Island, U. S. A. (MacMahon, Pugh and Ingalls, 1953), in France (Frezal, Kelley, Guillemot and Lamy, 1964), or recently in Liverpool (Smithells, 1964), and it is reported that none exists in Japan. No explanation of this apparent seasonal influence is available. It has not been possible to relate it to epidemic infections some 7 months before the births. No seasonal trend has been reported with spina bifida.

A seasonal incidence has also been reported for congenital dislocation of the hip independently in Hungary, Germany, Japan, and England (Andren and Palmen, 1963). In the English series from Birmingham (Record and Edwards, 1958), the ratio of winter to summer births for 186 patients was 1.6 and in a series of 219 cases from London (Carter and Wilkinson, unpublished) the ratio was again 1.6. A plausible explanation of this is that the mechanism is postnatal and related to the tighter swaddling of the infant in winter. The same mechanism, in a more extreme form, is thought to be responsible for the high incidence in Lapps and North American Indians. The recent Swedish data of Andren and Palmen (1963) lend some support to this view. In a series of 816 cases from areas in which the diagnosis of congenital dislocation of the hip was made soon after birth, there was a small excess of summer births, but in other areas in which patients were diagnosed mostly after the age of 1 year, there was a small excess of winter births.

Birth Order

Birth order effects have been reported for anencephaly, congenital dislocation of the hip, talipes equinovarus and pyloric stenosis. The most marked birth order effect is probably that seen with congenital dislocation of the hip. This appears to have first been noticed as long ago as 1909 by Dreesmann (quoted by Andren, 1962) who found 48 per cent of a series of 139 were firstborn at a time when only about 33 per cent of children would have been expected to be firstborn. Record and Edwards (1958) found 50.4 per cent of 186 cases in Birmingham were firstborn, compared with 34.4 per cent of a control series, a relative incidence of 1.5. The proportion of firstborn in the controls in this series is surprisingly low for a recent English series, nevertheless there is

clearly an excess of firstborn. In a series of 187 cases from The Hospital for Sick Children in London (Carter and Wilkinson, unpublished) 100, that is 53.5 per cent, were firstborn when the expected figures for London for the same period would have been about 40 per cent, a relative incidence of 1.3. While in none of these series is the number of observations large enough for differences to be highly significant, they are consistent. The explanation of the relationship with congenital dislocation of the hip is in part at least the role, as mentioned below, of intrauterine malposition.

A definite excess of first births has also been shown for anencephaly and spina bifida in Birmingham (Record and McKeown, 1949), Rhode Island (Ingalls, Pugh and MacMahon, 1954) and in the Scottish data (Edwards 1958). There is a suggestion also that there is an excess at birth orders of five or more. No explanation of the excess of first births and perhaps late births is available. The social class effect might account for an excess of late birth orders, but not for an excess of first births.

A birth order effect for infantile pyloric stenosis was first suggested by Still (1927). Still was unfortunate in his choice of controls, mostly children with bronchopneumonia, a condition which occurs particularly in children in large families in poor circumstances. Nevertheless the proportion he found, 48.5 per cent firstborn, is certainly high for that time in London. The birth order effect is not large in modern series, but there is some evidence that there is an excess of firstborn among patients in whom the symptoms present relatively late (McKeown, MacMahon and Record, 1952).

Parental Age

Marked maternal age effects are known for the trisomy 21 syndrome and other trisomies, and paternal age effects have been shown for cases of achondroplasia and acrocephalosyndactyly, due to fresh mutations. However, no clear parental age effects independent of birth order effects have been shown for the common malformations. Palmer (1964) from Indianapolis has reported a reduced mean maternal age for talipes equinovarus, but this has not been confirmed in a series from southwest England (Wynne-Davies, 1964). However, recently, both Fraser and Calnan (1961) and Woolf (1963) have found a small paternal age effect in harelip (± cleft palate).

Intrauterine Mechanical Effects and Breech Births

Intrauterine mechanical effects are certainly important in the etiology of congenital dislocation of the hip and probably for talipes equinovarus.

It has been shown experimentally (Wilkinson, 1963) that if one leg of a young female rabbit is splinted in the position of hip flexion, knee extension and lateral rotation of the leg, and the ligaments are relaxed by injections of estrone followed by progesterone, the hip undergoes an traumatic dislocation.

In man the frank breech malposition involves hip flexion and knee extension, and the association of breech birth with congenital dislocation of the hip has long been known. In the firstborn, breech births are nearly always frank breeches and it is particularly firstborn breeches which are associated with the malformation. In a London series, 27 of 183 (Carter and Wilkinson, 1964; unpublished observations) were breech born, that is 15 per cent, and a further 9 per cent had undergone version late in pregnancy. Of the 27 breech births, 20 were firstborn and it was known that of the remaining seven at least two were frank breeches. There is, therefore, a clear indication that this malposition predisposes to breech malformation. The dislocating position of the legs could well be associated with a vertex presentation, but would then usually not be noted as abnormal.

Genetic Factors

Sex Ratio

A clear indication of the importance of genetic factors in the common malformations comes from their sex ratio. This male:female ratio varies from about 5:1 for pyloric stenosis, to 2:1 for talipes equinovarus, 1.5:1 for harelip (± cleft palate), 1.5:1 for spina bifida cystica, 1:2 for anencephaly and 1:6 for congenital dislocation of the hip.

There is no suggestion from family studies that genes on the X-chromosome play a direct part in the causation of any of these conditions; for example, it is not at all uncommon for males who have had pyloric stenosis to have affected sons. The sex ratios are best interpreted as due to partial sex limitation. Mechanisms are not yet clear, but the female preponderance of congenital dislocation of the hip is probably due to the greater sensitivity of the female fetus to relaxing effects of estrone and progesterone on periarticular ligaments (Wilkinson, 1963).

In several conditions it is possible to show that there is a significant relationship between the sex of the index patient and the incidence of the condition in first degree relatives.

Family Patterns

Neither twin studies nor the sex ratio provides much information about the type of inheritance involved in the common malformations;

but some indication of the type is given by the family patterns. Family studies are gradually becoming available in which: (a) the index patients are a representative sample of all patients with the condition; (b) the families have been visited, and the family history noted, by a trained worker, in their own homes; and (c) the diagnosis of abnormality in a relative has been confirmed by personal examination or properly documented from hospital records.

Harelip (± Cleft Palate)

The most complete and extensive family studies available are those for harelip (± cleft palate) uncomplicated by other major malformation and not part of a known syndrome. The condition, except in the severest forms, has a low mortality and diagnosis is simple. In 1942 Fogh-Andersen showed that while the over-all incidence of the malformation in Denmark was close to one per thousand, the incidence in full-sibs was 4.9 (± 1.0) per cent, aunts and uncles 0.8 (± 0.1) per cent and first cousins, 0.3 (± 0.1) per cent. The incidence in sibs of index patients was not affected by the presence of another affected member in the family unless it was a parent that was also affected and then the incidence was, in this series, 8 in 59, or 14 per cent. Curtis, Fraser and Warburton (1961) from Montreal report essentially similar findings for incidence and proportion of sibs affected. In Woolf, Woolf and Broadbent's (1963) series from Utah, U. S. A., the population incidence and the proportion of relatives affected were again like those in the Danish series: sibs 4.6 (± 0.6) per cent, aunts and uncles 0.7 (± 0.1) per cent, nephews and nieces 0.8 (± 0.3) per cent and first cousins 0.4 (± 0.1) per cent. In addition, 7 of 164 children were affected (4.3, ± 1.6 per cent).

A further large series from London (Roberts, Carter and Buck, unpublished) gives some additional information because the index patients were chosen from among those treated from 1920 to 1940 at The Hospital for Sick Children, thus providing information on a relatively large number of children, as well as sibs, of index patients. However, since this series was limited to those index patients who had children, the more severe degrees of malformation are less well represented than those in the Danish, Montreal, or Utah series, and this may account for the rather lower incidence found in the sibs and children; there is evidence, given below (see Table 4), that the risk, at least to first degree relatives, is affected by the severity of the malformation in the index patient. The data on sibs and children in the London series are summarized in Table 2.

TABLE 2—*Proportion Affected of First Degree Relatives of Index Patients with Harelip (± cleft palate)—London Series*

	Brothers	Sons	Sisters	Daughters	Total
184 Male index patients	9/273	6/183	9/251	2/171	26/878
	3.29%		2.61%		2.96%
107 Female index patients	2/118	9/114	5/132	0/97	16/461
	4.74%		2.18%		3.47%
Total	11/391	15/297	14/383	2/268	42/1339
	3.78%		2.46%		3.1%

The over-all incidence of the malformation in sibs in the London series is 25/774 (3.2 ± 0.6 per cent). The incidence in children, 17/565 (3.0 ± 0.7 per cent), is very close to that in the sibs. This rules out any substantial degree of recessive inheritance and also makes it unlikely that any factors in the intrauterine environment which persist from one pregnancy to the next are important in the etiology of the malformation. The finding in all these series that the proportion of parents affected is lower than that of sibs may be attributed to the lower reproductive fitness of those affected; in general the proportion of parents affected is a less satisfactory parameter than that of children affected.

In the London series, the incidence in aunts, uncles, nephews and nieces, taken together is 22/3525 or 0.6 ± 0.1 per cent, and for first cousins, 7/3518, 0.2 ± 0.1 per cent, very similar to those in the Danish and Utah series except for the rather higher figure in the latter for cousins. The figures for each type of relative in the Danish, Utah and London series are summarized in Table 3.

Relating these incidences in relatives to the incidence in the general population of about one in a thousand, first degree relatives have 30 to 40 times, second degree relatives six to seven times, and third degree relatives only two to three times the population incidence of this malformation.

It has been noted (Carter, 1961a) that for a condition with an incidence of about one in a thousand this sharp fall-off in the proportion affected, as one passes from first to second (and a smaller fall-off as one passes from second to third degree relatives), is very different from the even halving of the proportion affected with each decrease in the degree of relationship that one would expect if a dominant mutant gene (with reduced penetrance) was concerned. It is, however, to be expected if the genetic predisposition to develop the malformation is

TABLE 3—*Percentage Affected of First, Second and Third Degree Relatives of Index Patients with Harelip (± cleft palate)*

	Sibs	Children	Aunts and Uncles	Nephews and Nieces	First Cousins
Copenhagen	4.9 ± 1.0	—	0.8 ± 0.1	—	0.3 ± 0.1
Utah	4.6 ± 0.6	4.3 ± 1.6	0.7 ± 0.1	0.8 ± 0.3	0.4 ± 0.1
London	3.2 ± 0.6	3.0 ± 0.7	0.6 ± 0.2	0.7 ± 0.3	0.2 ± 0.1

multifactorial, in the sense of being due to the combined action of many genes, a concept that Record and Edwards (1958) had already advanced in connection with congenital dislocation of the hip. If it is presumed that those at one end of the normal distribution of the genetic predisposition are at risk, then a substantial proportion of first degree relatives (with a distribution for the predisposing genotype about a mean halfway between the population mean and that of affected index patients) will be at risk, but considerably fewer second degree relatives and fewer still third degree relatives. Distribution of the latter would be about a mean deviating from the population mean by only one-eighth the deviation of the affected and index patients. It might be argued that a dilution of the environmental factors, which must also play a part as one passes from first to second to third degree relatives, could also produce a fall in incidence of the same type. However, the regularity of the pattern in different series from different Caucasian populations, and the very similar proportion of, for example, aunts and uncles and nephews and nieces affected, even though they are two generations apart and often born in different centuries, suggests that the genetic relationship is the important one in determining the proportion affected of relatives of different degrees. If this is the case, the grandchildren of index patients, when they come to be born, will also show an incidence similar to that in other second degree relatives, and it will be interesting to see if this proves correct.

On a multifactorial hypothesis of this kind one might expect that the relatives of the less frequently affected sex and of the more severely affected patients are more often affected. With harelip (± cleft palate) the sex ratio of less than two would not imply any great increase in the proportion of affected relatives of female compared with those of male index patients. For first degree relatives in the London series the difference is not significant, 3.47 per cent, compared with 2.96 per cent, but it is in the direction expected.

As regards severity, in the London series there is, for the first degree relatives, a clear gradation, as shown in Table 4, which is significant at the 0.05 level, the χ^2 for trend (1 degree of freedom) being 4.8. A

similar trend, but not significant at the 0.05 level, is seen in the Danish series, and combining the series the trend is highly significant, χ^2 being 7.97. The proportion of first degree relatives affected falls from 5.74 per cent for index patients with double harelip and cleft palate, to 4.21 per cent for unilateral harelip and cleft palate, to 2.46 per cent for unilateral harelip without cleft palate (cases of double harelip without cleft palate were too few to provide useful information).

Pyloric Stenosis

The family patterns for pyloric stenosis again indicate multifactorial genetic determination (Carter, 1961a, 1961b). The most recent data (Carter, 1961b; Carter and Evans, unpublished) on first degree relatives in series of index patients treated operatively at The Hospital for Sick Children from 1920 to 1939 and who have had at least one child (normal or abnormal) are summarized in Table 5, together with a supplementary series of data on women patients who have had no children or were treated in 1940–1949.

As with harelip (± cleft palate) the proportion of children affected is at least as high as that of sibs affected, making it unlikely that either recessive inheritance or any persistent feature of the intrauterine environment is important in the etiology of the condition. The apparently higher incidence in children than sibs, is not readily explicable on genetic grounds, but, if more than a random fluctuation, is probably due to more complete diagnosis of pyloric stenosis in recent years.

Infantile pyloric stenosis is a self-limiting disorder; the patient if untreated either dies or recovers completely at about the age of 4 months. If strict criteria of diagnosis are adopted the ascertainment of cases among more remote relatives and among earlier generations, for example, aunts and uncles, will be far from complete. Arguments from the ratio affected in first, second and third degree relatives are best based on relatives who are contemporary or younger than the index patients. The main London series has, so far, given a proportion affected of 31 in 352 sons and 14 in 334 daughters, 11 in 278 brothers and 7 in 287 sisters, 6 in 275 nephews, and 1 in 265 nieces, 8 in 1,299 male first cousins, and 3 in 1,270 female first cousins, compared with a population incidence of about 5 per 1,000 for male live births and 1 per 1,000 for female live births. The number of nephews, nieces and first cousins affected in the series is still small; the data indicate, however, a sharp fall in the incidence as one passes from first to second degree relatives: from about 20 times the population incidence in children to about 4 times in nephews and nieces, to only 1½ times in first cousins. The

TABLE 4—*Proportion Affected of Relatives (sibs + children) of Index Patients with Harelip (± cleft palate) According to the Severity of the Malformation in the Index Patient (index patients with double harelip without cleft palate are excluded)*

	Double Harelip + Cleft Palate	Single Harelip + Cleft Palate	Single Harelip
Copenhagen	13/223 5.8%	37/681 5.4%	6/211 2.8%
London	13/230 5.7%	17/601 2.8%	1/480 2.3%
Total	26/453 5.74%	54/1282 4.21%	17/691 2.46%

TABLE 5—*Proportion Affected of Relatives of Index Patients, Treated by Rammstedt's Operation—Main London Series, Treated in 1920–39 and Having at Least One Child*

	Brothers	Sisters	Sons	Daughters	Nephews	Nieces	Cousins male	Cousins female
Male index patients (281)	5/230	5/242	19/296	7/274	5/231	1/213	6/1061	3/1049
Female index patients (61)	6/48	2/45	12/56	7/60	1/44	0/52	2/238	0/221
Total (342)	11/278	7/287	31/352	14/334	6/275	1/265	8/1299	3/1270
Supplementary London Series of Female Index Patients								
Female index patients (88)	5/53	7/56	2/5	0/2	3/16	0/26	4/507	2/473
Total female index patients	11/101	9/101	14/61	7/62	4/60	1/78	6/745	2/694

figures for cousins belong, however, on average, to one generation earlier than the children and nephews and nieces. The pattern is entirely consistent with multifactorial inheritance.

If the genetic predisposition is essentially the same in males and females, and is multifactorial, the high sex ratio would imply that affected females are more extreme deviants than the males and this should be reflected in the incidence in first degree relatives. This is in fact seen clearly in the London series, in which the main series has been supplemented by including family data on women treated in 1920–1939 who have not yet had children and women treated in 1940–1949 (see Table 5). Combining the first degree relatives, the proportions affected for the relatives of male index patients are 4.6 ± 1.0 per cent for males, 2.3 ± 0.7 per cent for females; and for relatives of female index patients are 15.4 ± 3.1 per cent for males and 9.8 ± 2.4 per cent for females. These are, respectively, approximately 10, 25, 30 and 100 times the incidences in the same sex in the general population; a progression entirely to be expected on multifactorial inheritance.

Talipes Equinovarus

Family patterns for talipes equinovarus (excluding cases secondary to other malformations) have been reported by Wynne-Davies (1964; unpublished observations). She defined the condition as that in which there is plantar flexion of the ankle and inversion of the foot, and, excluding the mild cases that recover without treatment, found that the incidence of the condition in the Exeter area was 1.24 (males 1.6, females 0.8) per thousand live births.

In the Exeter series, the proportion affected with the same type of talipes equinovarus in first degree relatives (parents and sibs combined) was 2.1 ± 0.7 per cent, in second degree relatives (aunts and uncles) 0.61 ± 0.3 per cent, and in first cousins 0.2 ± 0.1 per cent. This indicates approximately a twenty-fold increase in first degree, fivefold increase in second degree, and twofold increase in third degree relatives compared with that of the general population of the area.

The sex ratio was two males to one female and subdividing the index patients by sex, for male index patients, 5 of 212 (2.4 per cent), of male and 0 of 187 female first degree relatives were also affected. For female index patients, 5 of 80 (6.3 per cent), of male and 2 of 81 (2.5 per cent), female first degree relatives were affected. If male and female relatives are combined, even though the sex ratio is only two, the excess in the proportion of relatives of female index patients similarly affected is just significant at the 0.05 level.

TABLE 6—*Proportion Affected of Relatives of Index Patients with Anencephalus and Spina Bifida Cystica—Southampton Series*

	Brothers	Sisters	Father's Brothers	Father's Sisters	Mother's Brothers	Mother's Sisters	Father's Brothers' Children	Father's Sisters' Children	Mother's Brothers' Children	Mother's Sisters' Children
Anencephalus (27)	0/23	2/18	0/50	0/43	0/47	0/43	0/32	0/34	0/30	5/60
Spina-Bifida (59)	3/66	4/55	0/97	0/95	0/114	3/107	1/64	0/98	0/116	3/147

A recent American series for Indianapolis (Palmer, 1964) gives a rather higher proportion of sibs, 14/283 (4.9 per cent), affected and also shows 3/110 fathers and 10/110 mothers affected; but the report does not indicate just how the index patients were selected. In addition, 16 aunts and uncles, and 16 first cousins were affected, but the total number of aunts, uncles and first cousins surveyed in this series has not yet been reported.

Anencephaly and Spina Bifida

With anencephaly and spina bifida cystica, the only first degree relatives who provide genetic information are sibs, though increasingly patients with the milder forms of spina bifida cystica will survive and have children.

In Birmingham (Record and McKeown, 1950a; 1950b) between 1940–1947 the incidence of anencephalus was 2.3 per thousand live births, and of spina bifida cystica 2.7 per thousand live births. The proportion of sibs affected born *subsequent* to the first index patient in the family were: following an anencephalus, 4 in 190 (two with anencephalus and two with spina bifida), 2.1 per cent: following a child with spina bifida cystica 8 of 205 (six with spina bifida and two with anencephalus), 3.9 per cent. This proportion in subsequent sibs will be rather less than the proportion of sibs affected, because of the primiparity effect.

In Southampton (Williamson, 1965) where the incidence of anencephalus in the total population was 2.0, and of spina bifida cystica 3.2, per thousand total births, the proportion affected of sibs of index patients with anencephalus was 2 in 41, 4.9 per cent (both with anencephalus), and of index patients with spina bifida cystica 7 in 121, 5.9 per cent (one with anencephalus and six with spina bifida cystica). Her findings are shown in Table 6.

In Rhode Island, U.S.A. (MacMahon, Pugh and Ingalls, 1953), the incidence of anencephalus was 1.9 and of spina bifida 2.5 per thousand total hospital births. The incidence in births subsequent to the first index patient was, after anencephalus, 6 in 119, 5.0 per cent (two anencephalus, three spina bifida, one "hydrocephalus") and after spina bifida cystica 11 in 215, 5.1 per cent (five anencephalus and six spina bifida cystica).

Thus it would appear that in these areas the incidence of anencephaly or spina bifida cystica in the sibs of anencephalics is about six times the population incidence; that in the sibs of patients with spina bifida cystica the proportion affected with spina bifida cystica or anencephaly is about 10 times the population incidence, and the major part of the risk

is of the recurrence of the same malformation. Taking both malformations together the proportion of sibs affected is about eight times the population incidence.

In these series information on other specified relatives is given in detail only in the Southampton study, and even here the proportion of aunts and uncles affected will inevitably be under-represented if strict criteria are adopted in accepting a relative as affected. This will be true, but to a lesser extent, for the contemporary first cousins. In the Southampton series, for anencephaly 0 in 183 aunts and uncles, and 5 in 156 first cousins were affected with anencephaly or spina bifida cystica; for spina bifida cystica 3 in 413 aunts and uncles, and 4 in 425 first cousins had anencephaly or spina bifida cystica. Taking both malformations together the proportion of aunts and uncles documented as being affected (almost certainly an underestimate of the true proportion) is no more than the population figure, but the incidence in first cousins is about twice the population incidence.

With a sex ratio of one in two, it might be expected that there would be a small increase in the proportion of sibs affected of the male index patients, but the available series are not yet large enough to test this.

While the family patterns for these malformations are compatible with multifactorial genetic determination, the familial concentration is appreciably less than in the other conditions surveyed above.

The possible importance of recessive genetic factors or of a persistent intrauterine environmental factor cannot be assessed until there is information on the proportion of the children of index patients with spina bifida cystica. However, there is already an indication of a possible genetic factor acting through the intrauterine environment. Record and McKeown (1950a) quote the surveys of Hindse-Neilson (1938) and Murphy (1950) as suggesting that relatives on the maternal side might be more often affected than those on the paternal side, but noted that this might be because the mother is often the only informant. In the Southampton series, however, there was a striking concentration of cases on the maternal side; for anencephalic index patients no aunts and uncles were proved to be affected, but five affected cousins were mother's sister's children; for spina bifida index patients, all three affected parental sibs were mother's sisters and, of the four affected cousins, three were mother's sisters' children and one was a father's brother's child. If this relatively high risk to the children of female relatives of the mother is confirmed in further series, it will suggest that some women are genetically predisposed to provide an intrauterine environment which favors the development of anencephaly or spina bifida cystica in their

children. There are in fact no clear indications at present that a man can carry any genetic factors which predispose to anencephaly or spina bifida cystica in his children.

Congenital Dislocation of the Hip

No large scale modern family studies of congenital dislocation of the hip are yet available. Record and Edwards (1958) in a family study based on 164 index patients found the proportion of sibs affected to be 1 of 106 brothers and 10 of 116 sisters. Taking both sexes together this is approximately 50 times the population incidence. They also found 2 in 1,072 of aunts and uncles and 2 in 610 first cousins affected.

An as yet small series of patients first treated at The Hospital for Sick Children, London, between 1920 and 1939, a generation ago, comprising 80 female and 12 male index patients shows 0 of 96 brothers, 3 of 89 sisters, 0 of 51 sons and 5 of 52 daughters affected (Carter and Brown, unpublished). This supports the indication of an earlier London Orthopaedic Hospital series (Muller and Seddon, 1953) that children were at least as often affected as sibs and suggesting a total incidence in first degree relatives of about 30 times the population incidence of about 1 in a 1,000 live births. In this series, 0 in 97 nephews and 3 in 113 nieces were affected. The findings in the two series are summarized in Table 7.

Information on sibs only is also available from a more recent series of patients, 143 female and 21 male, treated at The Hospital for Sick Children between 1948 and 1958 (Carter, unpublished). Of the sibs of the female index patients, 1 in 115 brothers and 5 in 109 sisters were affected; of the sibs of the male index patients 4 in 17 brothers and 1 in 18 sisters were affected. In all, 11 in 259 sibs were affected, that is about 40 times the population incidence.

Combining the Birmingham and two London series, the incidence in first degree relatives (sibs and children) is 30 in 769, about 40 times the population incidence, in second degree relatives (aunts, uncles, nephews and nieces) is 5 in 1,282, about 4 times the population incidence, and in first cousins 2 in 1,398, about 1½ times the population incidence; considering only the female relatives of female index patients the corresponding relative incidences are 35, 3, and 2. This is consistent with multifactorial inheritance. As might be expected from the sex ratio there are indications that the near relatives of male patients are more often affected than those of female patients, but this needs confirmation.

Mention has been made of the importance of pre- and postnatal me-

TABLE 7—*Proportion Affected of Relatives of Index Patients with Congenital Dislocation of the Hip—Birmingham and London Series*

	Brothers	Sisters	Sons	Daughters	Uncles	Aunts	Nephews	Nieces	First Cousins	
									Male	Female
Birmingham										
Male index patients (25)	0/14	1/14	—	—	0/84	0/91	—	—	0/66	0/55
Female index patients (139)	1/92	9/102	—	—	1/445	1/452	—	—	0/239	2/250
London (treated 1920–39)										
Male index patients (12)	0/11	1/15	0/3	0/7	—	—	0/16	1/13	0/23	0/39
Female index patients (80)	0/85	2/74	0/48	5/45	—	—	0/81	2/100	0/362	0/364

chanical factors in the development of congenital dislocation of the hip, and this condition is the only one of those under discussion in which there is some indication of the nature of the detailed mechanisms of the genetic factors concerned. Carter and Wilkinson (1964) in a London series found that about three quarters of the male patients and about one quarter of female patients had a generalized joint laxity of a degree which they found in only about 6 per cent of normal boys and girls. This joint laxity, which may also play a part in the genesis of talipes equinovarus (Wynne-Davies, 1964) often behaves as a dominant condition (Carter and Sweetnam, 1958; 1960) though, because of the subjective element in its evaluation and the difficulty in recognizing it in the elderly, the inheritance is not fully established. There are indications that a temporary perinatal hormonal joint laxity in infant girls substitute for persistent joint laxity and is responsible for the ratio of one boy to six girls affected with congenital dislocation of the hip.

A second genetic predisposition concerns the development of the acetabular socket of the hip joint. Some years ago Faber (1937) showed that in unaffected relatives of patients with congenital dislocation of the hip it was not uncommon to find indications of shallow acetabula combined with a tendency to early osteoarthritis. Such acetabular dysplasia cannot be assessed on the size of the dislocation because if the head of the femur is not in the acetabulum the latter does not develop normally. It is present, however, in nearly one half of unilateral cases on the unaffected side (Wilkinson and Carter, 1960). When present it may also be seen in one or both of the unaffected parents. No quantitative genetic studies of acetabular dysplasia are yet available, but it is probable that the inheritance will prove to be multifactorial.

Discussion

The family patterns of the four malformations, harelip (± cleft palate), congenital dislocation of the hip, talipes equinovarus and pyloric stenosis show a similarity. This is illustrated in Table 8.

The somewhat lower relative incidences for pyloric stenosis are to be expected since this malformation has a population incidence two to three times higher than the other three. But girl index patients have an incidence of about one in a thousand and if females only are considered the relative incidences are even higher than for the three other malformations. The family patterns for the still more common central nervous system malformations (if spina bifida and anencephaly are taken together) are less well established, but seem likely to have very

TABLE 8—*Proportion Affected of First, Second and Third Degree Relatives of Some Common Malformations, Relative to the Incidence in the General Population*

	Harelip (± Cleft Palate)	Congenital Dislocation of the Hip		Talipes Equinovarus	Pyloric Stenosis		Anencephalus and Spina Bifida
		All Patients	Female Relatives of Female Patients		All Patients	Female Relatives of Female Patients	
Population incidence (approximate)	0.001	0.001	0.0018	0.001	0.003	0.001	0.005
Monozygotic twins	x 500	x 500	x 300	325	150	—	—
First degree relatives	x 35	x 40	x 35	20	20	100	8
Second degree relatives	x 7	x 4	x 3	5	4	12	—
Third degree relatives	x 3	x 1½	x 2	2	1½	3	2

much the same form as the others, but with the expected lower relative incidences.

It would be artificial to try and fit any particular model of multifactorial inheritance too closely to these relative incidences. It is worth noting, however, that on Gruneberg's concept of quasi-continuous variation (Gruneberg, 1951) if the mean endowment, of those affected, for an underlying multifactorial genotype predisposing to the malformation, were beyond a threshold 2¾ standard deviations from the population mean, and about a third of the three per thousand beyond this threshold were clinically affected, this would give a population incidence of about one in a thousand. The mean endowment of those affected would be about three standard deviations from the population mean, and the proportion of first, second and third degree relatives affected, would be approximately 35, 7 and 3 times the population incidence. If the mean endowment of those affected was a little more than 3 standard deviations from the population mean, as it would be if penetrance increased with distance beyond the threshold, the proportion of relatives affected would be higher but show the same kind of relationship.

Edwards' formulae, on slightly different assumptions (1960; personal communication) of $\rho^{2/5}$, $\rho^{2/3}$ and $\rho^{4/5}$ for first, second and third degree relatives, where ρ is the population incidence gives 60, 10 and 4 times the population incidence where ρ is one in a thousand.

Falconer (1965) has shown, on some not unreasonable assumptions, that relative incidences of this kind indicate high degrees of heritability in the population concerned. Applying his methods and considering first degree relatives the approximate heritability estimates would be nearly 100 per cent for pyloric stenosis in girls, about 70 per cent for pyloric stenosis in boys, about 80 per cent for congenital dislocation of the hip, about 60 per cent for talipes equinovarus, and about 60 per cent for anencephalus and spina bifida. Falconer points out, however, that both dominance and common family environment would raise these heritability estimates above the true value, and that this effect would be most marked with sibs. However, with the common malformations there are no indications that the estimates from sibs are higher than those from children. Also estimates based on second and third degree relatives, where common family environment will be less important, are compatible with those based on first degree relatives though because of the small numbers of the more remote relatives affected, the estimates based on them have wide limits of confidence.

The demonstration that the genetic predisposition to the development of a disorder is multifactorial is, of course, only a first step to the dis-

covery of the individual genes and the mechanisms by which they act and interact with the environment. Only in the case of congenital dislocation of the hip have we some inkling of these genetic mechanisms and of their interaction with environmental factors.

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CHAPTER 4

Evolutionary Origins of Human Proteins

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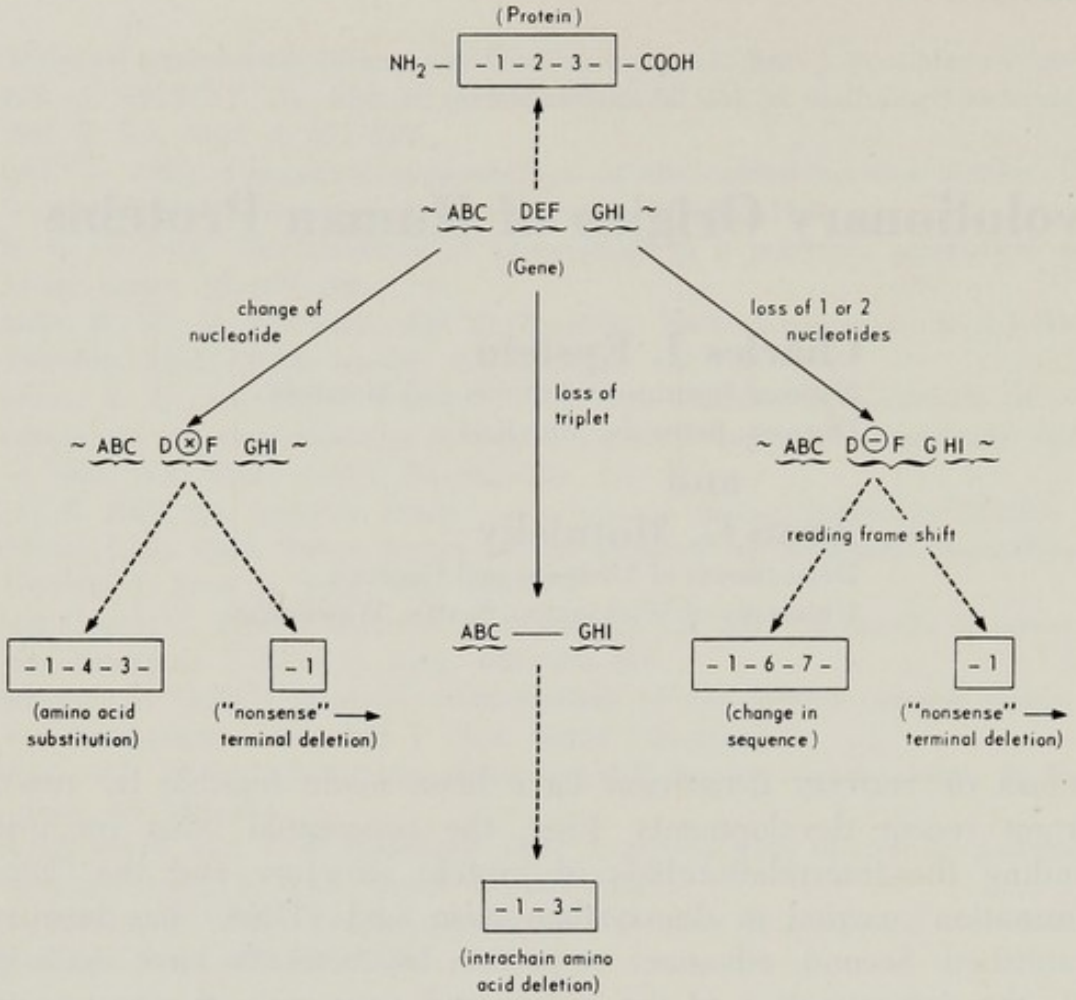
STUDIES OF PROTEIN EVOLUTION have been made feasible by two important recent developments. First, the conceptual basis for understanding the interrelationships of protein structure and the "genetic information" carried in desoxyribonucleic acid (DNA) has been well established. Second, advances in protein biochemistry have made possible the determination of the amino acid sequences of many proteins and polypeptides. By the examination and comparison of such sequences it is now possible to infer some of the genetic events involved in their evolution. A consideration of such events is especially pertinent for human geneticists since much of the impetus for the investigation of the evolution of human proteins was derived from the work on normal and abnormal human hemoglobins and from the studies of the various forms of haptoglobin. Anfinsen, in his book, "The Molecular Basis of Evolution," (1959) and Zuckerkandl and Pauling (1962), in their paper on "Molecular disease, evolution, and genetic heterogeneity," were among the first to consider the problems to be discussed below.

In this paper, the following questions will be considered. How does a "primitive" protein, the amino acid sequence of which is determined by a specific structural gene, evolve into a more "modern" protein or proteins which may be more or less related to it in structure and function? What molecular or chromosomal events accompany the evolution of such proteins? What advantages might accrue to the organism as a result of these evolutionary changes? And, finally, what are the selective forces that determine the course of protein evolution?



PROTEIN MUTATIONS

FIG. 1—The amino acid sequence of a protein can be altered by a change of a nucleotide base or loss of nucleotides. However, if a triplet of bases in which one base has been changed no longer codes for an amino acid, synthesis of the polypeptide chain will stop at the point of alteration. Likewise, a "reading frame" shift may produce the same result, but, if synthesis can continue, all residues beyond the point of alteration will be changed. Since synthesis of a polypeptide chain proceeds from the amino- to the carboxyl-end (Canfield and Anfinsen, 1963), the changes or cessation of sequence would be expected to occur to the carboxyl-side of the nucleotide alteration. Single amino acid deletions by the simultaneous loss of whole triplets are unlikely, and such deletions are more likely to be the result of unequal crossing-over or breakage-and-reunion (Fig. 3).

The arabic numbers (1-2-3 . . .) stand for one of 20 amino acids. The triplets in capital letters (*ABC, DEF* . . .) designate specific triplets of bases in DNA which code for a given amino acid (i.e., *DEF* coding for 2). ⊗ stands for a single nucleotide substitution, ⊖ stands for loss of a nucleotide.

FATES OF A PRIMITIVE PROTEIN AND OF ITS GENE

Amino Acid Substitutions

The simplest alteration that can occur in a primitive protein is for one of its amino acids to be changed into another by a single ("non-

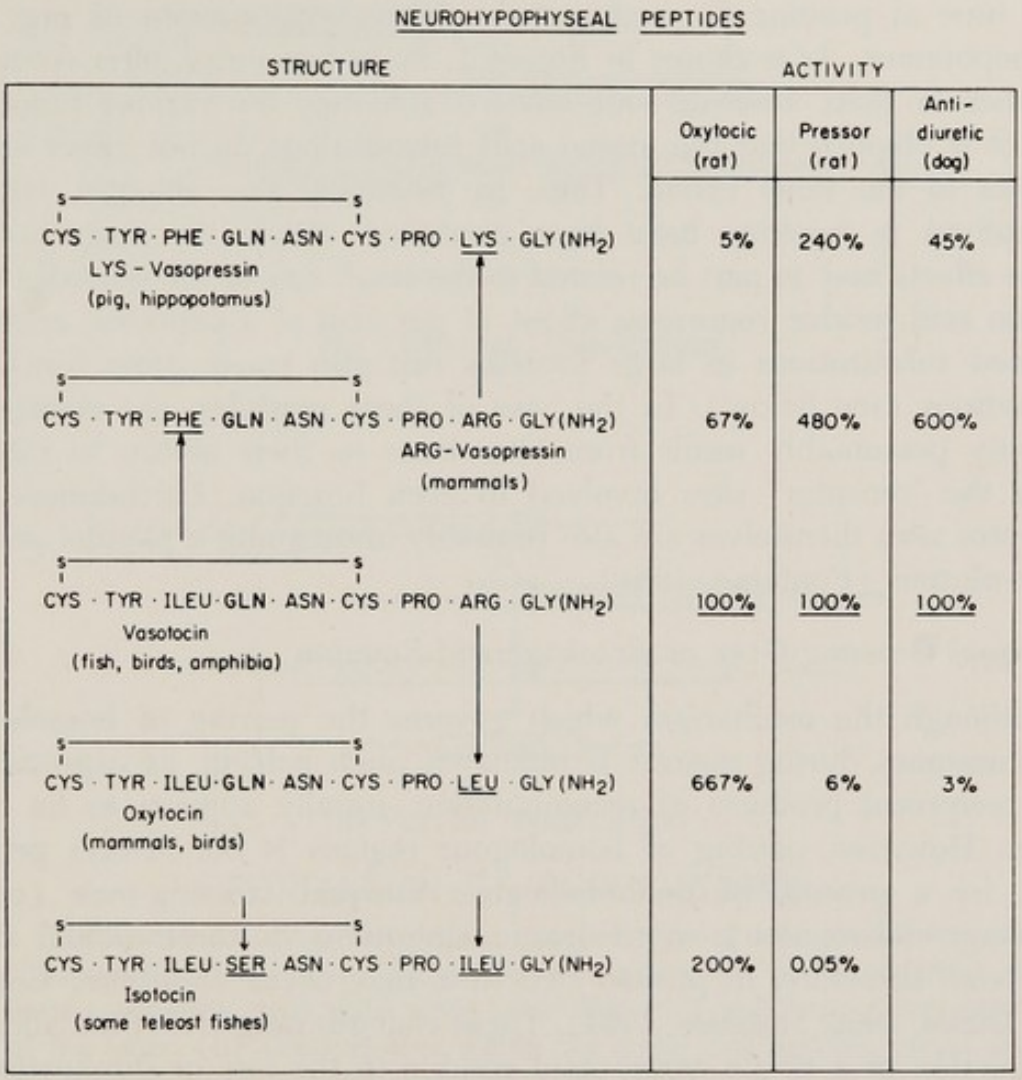


FIG. 2—Evolution of neurohypophyseal hormones by single-step amino acid substitutions. All activities are expressed relative to the activities of vasotocin in the various assay systems. Acher et al. (1964) have recently isolated a new hormone, mesotocin ($Ileu_8$-oxytocin), which they believe to be evolutionarily intermediate between isotocin and oxytocin.

sense") nucleotide base substitution (Fig. 1). This amino acid alteration, depending on its precise nature and location in the peptide chain, may have many functional effects. Several of these are well illustrated by the series of polypeptide hormones: vasotocin, arginine and lysine vasopressin, oxytocin, and isotocin (Acher, 1964) (Fig. 2). These peptides, despite their small size, appear to be synthesized in the same manner as larger polypeptides and proteins, and it will be assumed that their sequences are coded directly by structural genes. Vasotocin appears to have given rise to two other hormones by single site mutations. The conversion of arginine to leucine at position 8 resulted in oxytocin, while the substitution of phenylalanine for isoleucine at position 3 resulted in the arginine-8-vasopressin of mammals. A second mutation in the latter,

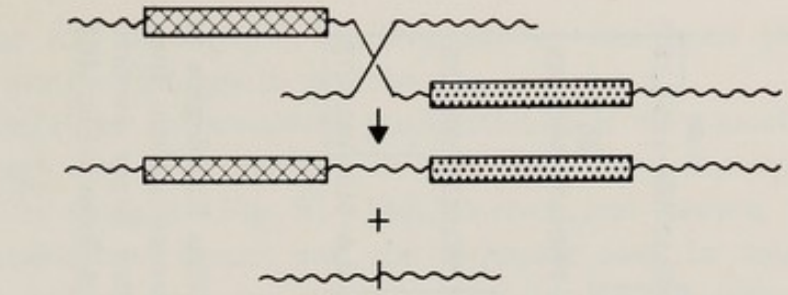
this time at position 8, produced the lysine-8-vasopressin of pigs and hippopotamus. As is shown in Figure 2, these hormones differ from one another in their absolute and relative activities for various functions, and it is obvious that the amino acid substitutions do not affect all activities to the same extent. Thus, by relatively few changes, marked alterations in function have been produced. While the magnitude of these effects may in part be related to the small size of the peptides (one amino acid residue represents about 11 per cent of a peptide), critically located substitutions in large proteins can also cause gross functional alterations (see below). In the case of these peptides, the changes in activity presumably result from alterations in their ability to interact with the "receptor" sites involved in each function. Furthermore, the receptor sites themselves are also probably undergoing a parallel process of evolution (Fontaine, 1964).

Unequal Crossing-Over or Breakage-and-Reunion

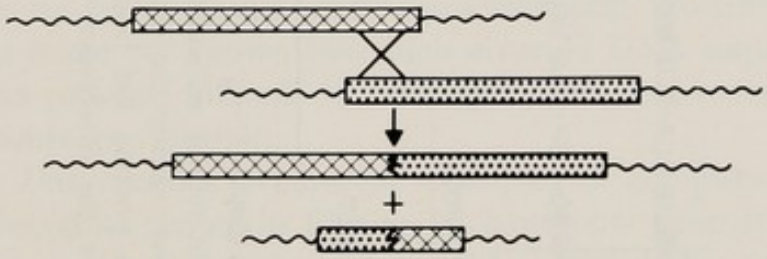
Although the mechanism which governs the pairing of homologous chromosomes during meiosis is unknown, such pairing, as expressed in the reciprocal products of recombination, usually appears to be quite exact. However, pairing of homologous regions is not always perfect, and, by a process of nonhomologous unequal crossing-over (or by breakage-and-reunion), more drastic alterations in chromosomal structure and, therefore, in protein structure, may occur (Smithies, Connell and Dixon, 1962; Smithies, 1964). These changes range from the addition or deletion of a single amino acid residue to the loss or duplication of an entire polypeptide chain (Fig. 3). Furthermore, as will be discussed later, the occurrence of a duplicated gene, by whatever process, will have other evolutionary consequences.

Amino Acid Deletions. A deletion of any size may be produced by unequal crossing-over (Fig. 3b). The deletion of a single amino acid has been "detected" by Braunitzer and Hilse (1963) in the α-chain of hemoglobin (Fig. 4). The human β-chain and the carp α-chain each resemble whale (and human) myoglobin in having five amino acid residues between phenylalanine and leucine (residues 42 and 48, respectively), but the human α-chain has only four. If it is assumed that the α- and β-chains have descended from a common myoglobin-like chain (see below), it is readily apparent by inspection of these sequences that the deletion in question (residue 46) occurred sometime after the evolution of the common precursor of the carp and human α-chains.

Partial Duplications. As is shown in Figure 3b, the counterpart of a partial gene deletion is a partial gene duplication. Direct evidence for



(a) Complete Duplication



(b) Partial Duplication

FORMATION OF DUPLICATIONS

FIG. 3—The effects of nonhomologous unequal crossing-over. (a) If the point of recombination lies between the paired identical genes, one of the daughter strands will have a complete gene duplication while the other will have a complete deletion of the locus. (b) Crossing-over within the genes will produce partial duplication in one strand and a partial deletion in the other.

such an occurrence has been obtained by Smithies, Connell and Dixon (1962) in the human haptoglobin system. The 2 α haptoglobin chain has been shown to represent an end-to-end fusion of the allelic 1 α F and 1 α S chains (which differ by one amino acid). Smithies (1964) now believes that a random chromosomal event (breakage and reunion), rather than unequal crossing-over, led to this new protein. The remarkable feature of the 2 α gene is that it not only codes for a polypeptide chain in which the 1 α (F and S) sequences are virtually intact, but also that the fusion α-chains are able to combine with β-chains to form protein molecules which preserve the original haptoglobin function. However, haptoglobin 1 (with 1 α chains) and 2 (with 2 α chains) do differ antigenically (Korngold, 1963) and possibly, to a small degree, functionally (Nakajima et al., 1963). It has been suggested (Smithies, Connell and Dixon, 1962) that haptoglobin 2-Johnson represents a partial triplication of the 1 α chain. This finding, if true, would

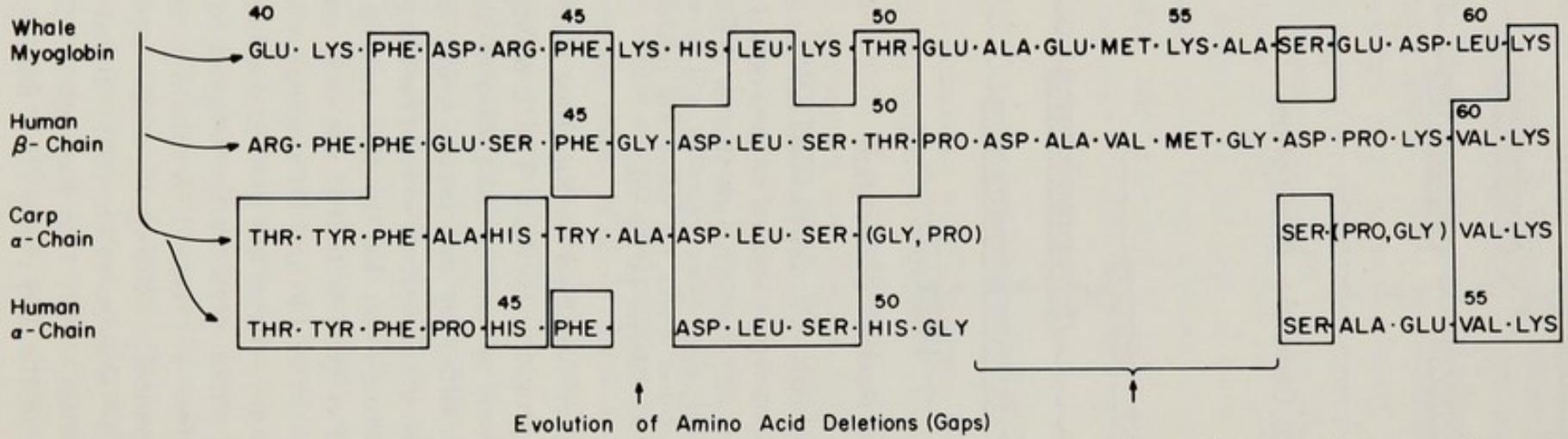


FIG. 4—Evolution of amino acid deletions. The carp hemoglobin α-chain retains an amino acid at a position homologous to residue 46 in whale myoglobin and the human hemoglobin β-chain. However, during the evolution of the human α-chain from the common human-carp α-chain precursor, the amino acid residue at this position has been deleted. Another larger deletion, common to both α-chains, is also apparent and presumably occurred after the divergence of the α and β-chains (Fig. 10) (Braunitzer and Hilse, 1963).

indicate that the hemoglobin-binding site(s) has been preserved in the face of drastic changes in protein size and shape.

The possibility of an *intragenic* duplication of a very localized nature has been suggested after analysis of a peptide from the cytochrome *c* (RHP) of *Chromatium* (Fig. 5) (Dus, Bartsch and Kamen, 1962). This peptide contains *two* hemes and the sequence may be interpreted as having been formed by a process of partial duplication followed by deletion of several amino acids from one of the duplicate segments. Therefore, the gene corresponding to this small region of the protein has been involved in several episodes of unequal crossing-over.

Smithies (1963) has also postulated the existence of *inverted* duplications which, he feels, would favor intrachromatid crossing-over. However, since it is not yet known how such inverted DNA sequences would code, it is, at present, difficult to demonstrate the existence of such inverted segments in a protein.

Complete Duplications. Numerous examples of complete gene duplication, produced as shown in Figure 3a, have now been recognized. In the initial example of simple amino acid substitutions, vasotocin was shown as giving rise to vasopressin and oxytocin, and, except in the lowest vertebrates, two hormones are always found: vasotocin or vasopressin and oxytocin or isotocin (Sawyer, Munsick and van Dyke, 1960; Acher, et al., 1962; Guttman, Berde and Stürmer, 1962). As another example, the ACTH, α-MSH, β-MSH group of pituitary peptide hormones may be cited (Fig. 6) (Schwyzer, 1963). Even though there are uncertainties about whether α-MSH is a product of a gene separate from that of ACTH and whether there are more than one β-MSH in some species (Burgers, 1963), it is clear that gene duplication has occurred at least once and possibly twice and that it has been followed by alterations in amino acid sequence, in chain length, and in activity.

A group of enzymes of particular interest in biochemical evolution is that of the esterases with an "active center" serine which reacts with diisopropylfluorophosphate (DFP). This reactivity has made possible the isolation of the serine-containing peptide from several proteins (Fig. 7). Their similarity in structure has prompted the suggestion that the active sites of these enzymes, particularly of trypsin, chymotrypsin, and elastase, might have evolved from a common precursor (Dixon, Kaufman and Neurath, 1958). It was not known whether gene duplication had actually taken place, or whether the "active center" peptide had somehow (e.g., by translocation, selection, or "convergent evolution") been incorporated into many diverse proteins. Now, with the announcement of the complete sequences of trypsinogen (Walsh and Neurath,

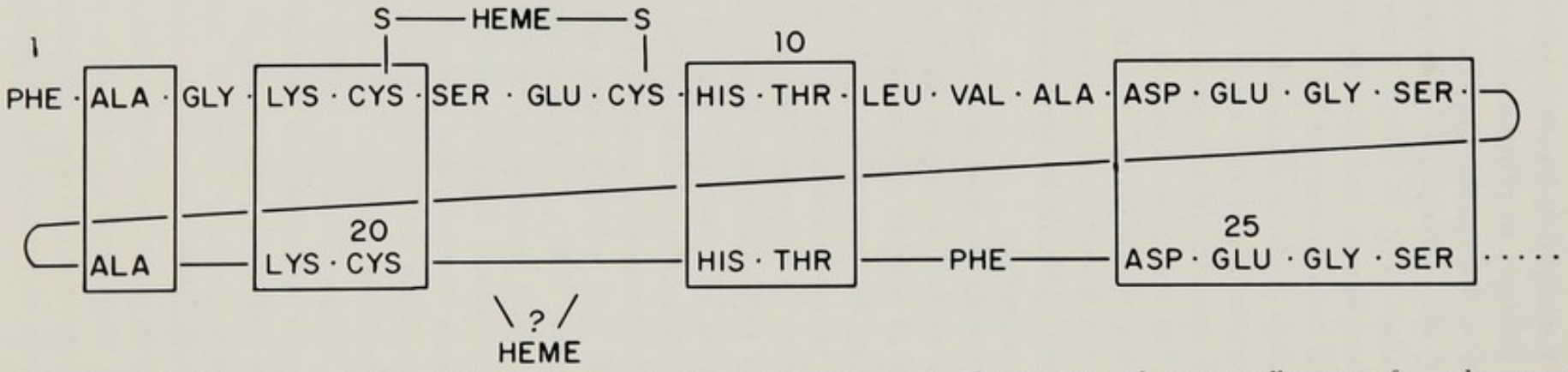


FIG. 5—Probable intragenic (partial) duplication in the cytochrome *c* of *Chromatium*. If proper allowance for subsequent deletions is made, several areas of sequence identity are apparent (after Dus et al., 1962).

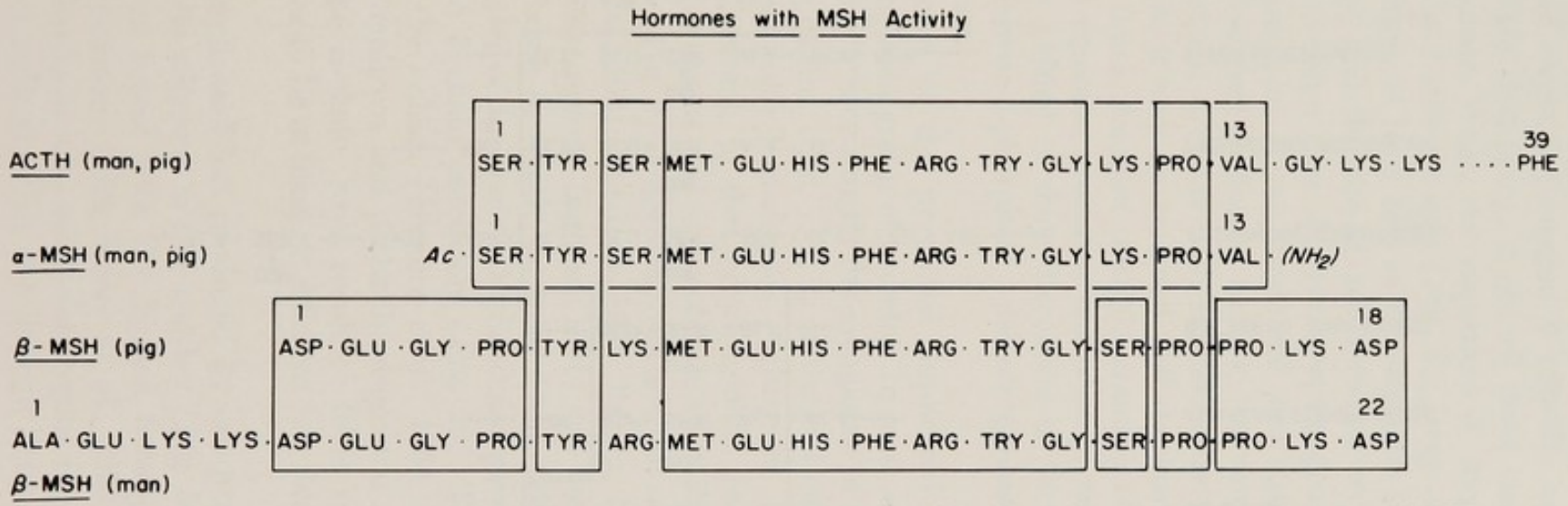


FIG. 6—Amino acid sequence homologies among the various hormones with melanophore-stimulating hormone (MSH) activity: ACTH, α-MSH, and β-MSH (Schwyzer, 1963).

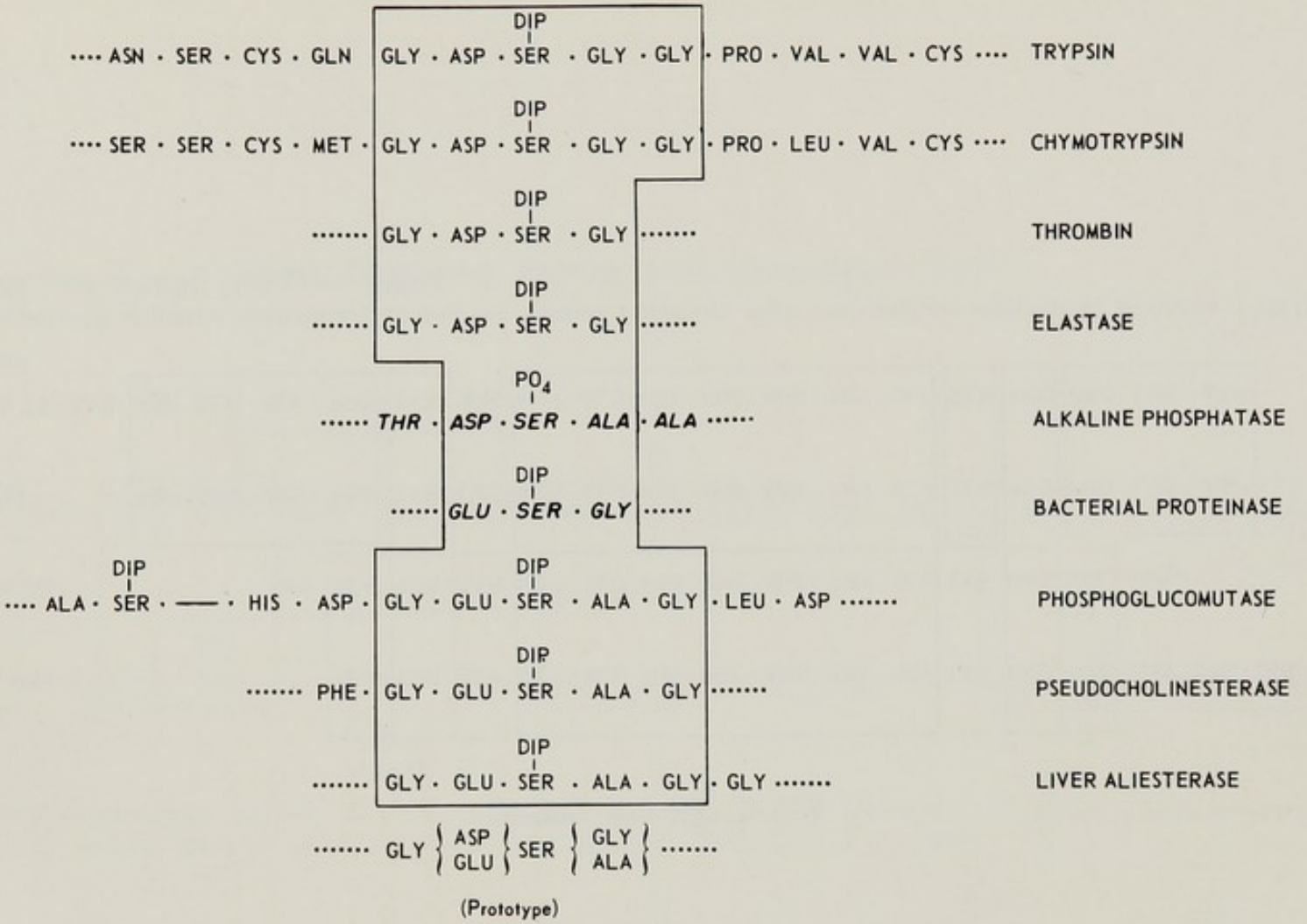
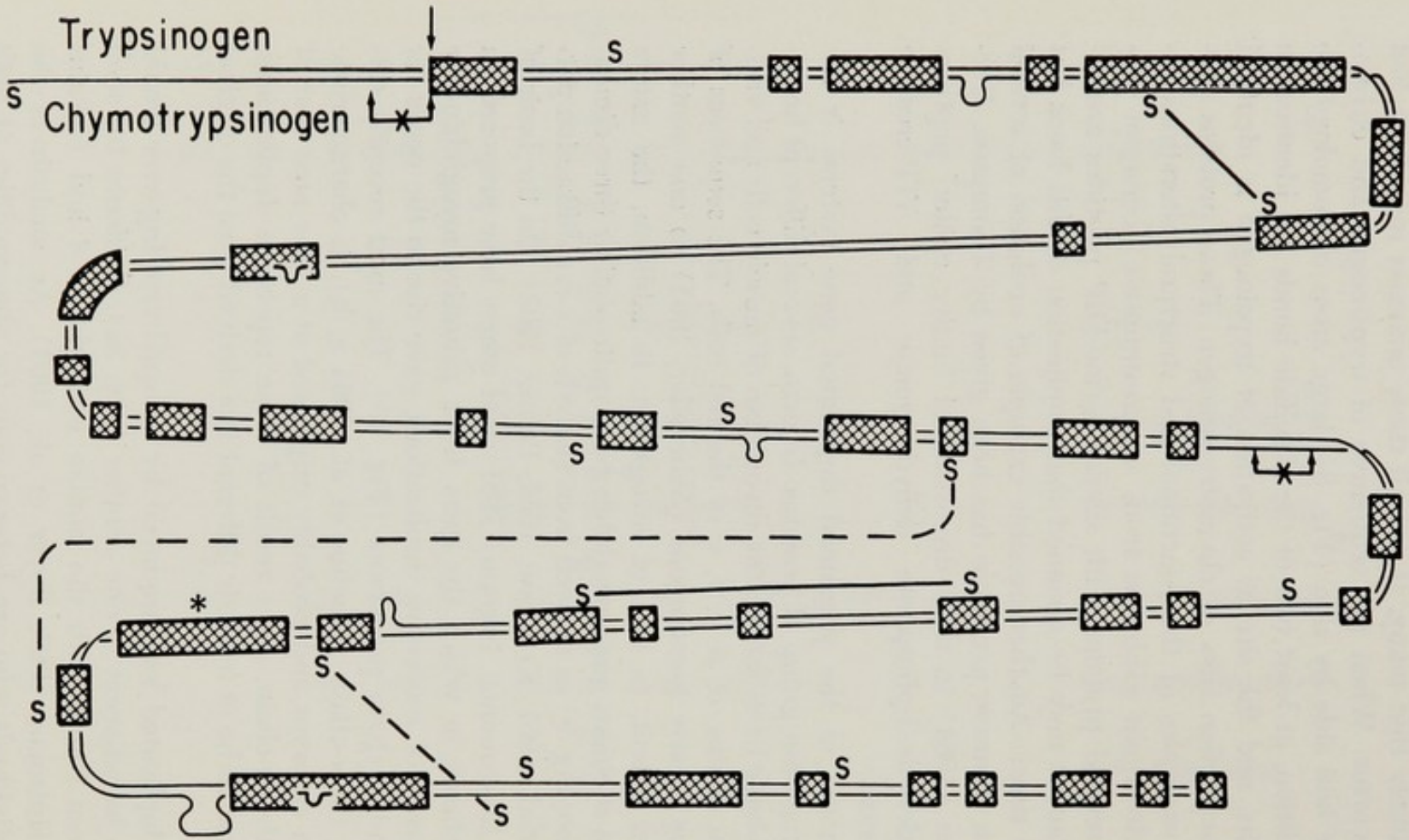


FIG. 7—Enzymes with reactive serines in the "active center." Two general groups of diisopropylfluorophosphate (DFP) inhibitible enzymes are recognized: those with the sequence GLY·*ASP*·SER·*GLY* and those with the sequence GLY·*GLU*·SER·*ALA*·GLY. In addition, there are two enzymes, bacterial proteinase and *E. coli* alkaline phosphatase, whose sequences appear to "bridge" those of the two groups (Schwartz et al., 1963; Dixon and Schachter, 1964; Bocchini, 1964; Hartley, 1964; Walsh and Neurath, 1964) (DIP-SER = diisopropylphosphorylserine; PO_4-SER = phosphoserine).

1964) and chymotrypsinogen A (Hartley, 1964) and partial sequences from elastase and chymotrypsinogen B (Smillie and Hartley, 1964), it appears quite likely that many, if not all these enzymes may have had a common precursor. When the sequences of trypsinogen and chymotrypsinogen are laid side by side (Fig. 8), large areas of homology are present. In addition, at least two of the disulfide bonds are identical in the two proteins, and the site of activation of trypsinogen is identical to one of the activation sites of chymotrypsinogen. These proteins provide excellent examples of the functional and structural alterations associated with divergent evolution from a (presumptive) common precursor. If the several proteins with similar serine-DIP peptides are indeed homologous, it may be assumed that duplication of this locus has occurred many times. Another possible example of evolution of several enzymes from a common precursor has been given by Thompson, Eveleigh and Miles (1964) in their discussion of "active center" peptides from glyceraldehyde-3-phosphate dehydrogenase and ATP-creatine phosphotransferase.

The best known of the presumed duplicated gene systems is the hemoglobin-myoglobin group of proteins. In man, several different hemoglobin proteins have been found: the myoglobin of muscle cells and various hemoglobin chains, α, β, γ, δ, ϵ, of the red cells. The sequences of these chains are clearly homologous (Schroeder, 1963) to one another and, to a lesser extent, to that of myoglobin. In addition, the native (folded) α and β chains and myoglobin have quite similar three-dimensional structures (Fig. 9) as derived from analysis of x-ray diffraction patterns (Cullis et al., 1961; Kendrew, 1962; Perutz, 1963). On the basis of the similarities in sequence, Ingram (1961) and others have proposed an evolutionary scheme in which the gene for a primitive myoglobin-like protein, by a series of successive duplications, gave rise to the genes for the α, γ, β, and δ chain precursors (Fig. 10). The most recently discovered chain, the ϵ-chain (Huehns et al., 1964 a, b), is characteristic of early human embryos and probably originated at a time near that of the origin of the α-chain. As a result of these repetitive duplications, there now appear to be *at least six* different loci derived from the original gene.

In general, duplicated loci produced by unequal crossing-over would be expected to be adjacent to one another (Fig. 3a). Evidence for such linkage has been found with the human hemoglobin β and δ-chains (Horton and Huisman, 1963; Boyer et al., 1963). An analysis of the offspring of individuals who are heterozygous for abnormalities at both the β and δ loci indicates that the linkage between these loci is quite



Sequence Homologies in Trypsinogen and Chymotrypsinogen

FIG. 8—Sequence homologies between trypsinogen and chymotrypsinogen. The blocks represent regions (approximately to scale) in which the proteins are identical in sequence. In addition, two disulfide bonds (solid lines) are identical in both, as is one of the sites at which activation by chain cleavage occurs (arrows). It is apparent that during the evolution of these proteins, various deletions have occurred in both polypeptide chains (Walsh and Neurath, 1964; Hartley, 1964).

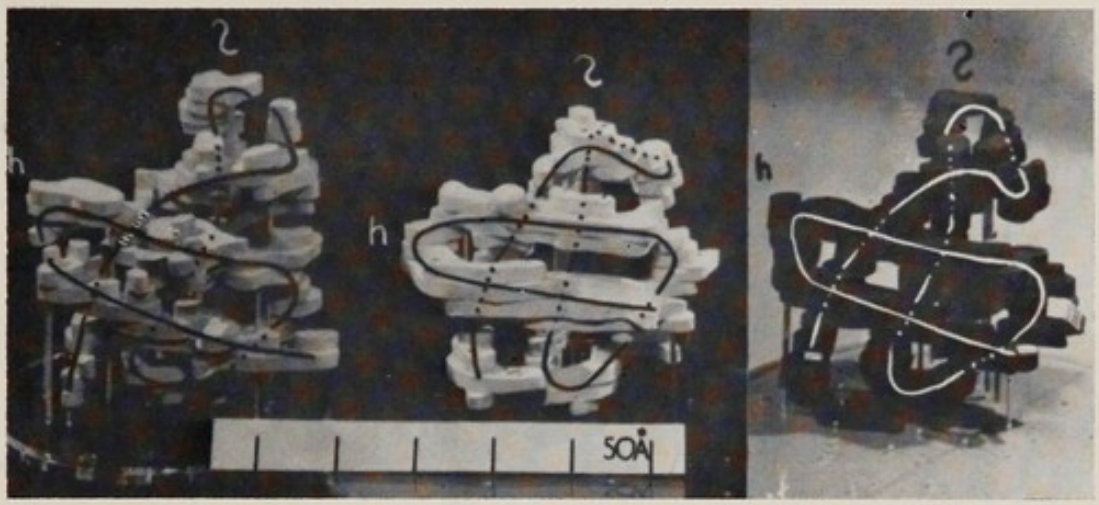


FIG. 9—The nearly identical three-dimensional configurations of sperm whale myoglobin and the α and β-chains of horse hemoglobin. Even with marked differences in amino-acid sequence, the configurations of these polypeptide chains have been remarkably preserved. (Reproduced. by permission, from Cullis et al., 1961.)

tight; no recombination has been detected in over 40 opportunities. Likewise, there are two β-chain components (differing by three peptide pairs) in the *diffuse* hemoglobin of certain strains of mice (Hutton et al., 1962a) (Fig. 11). These components are analogous to the human β- and δ-chains (which differ by four peptide pairs). In matings of mice possessing diffuse hemoglobin with mice possessing only a single β-chain, they were found to segregate as a linked unit (Hutton et al., 1962b). Linkage between the α and β-chain loci does not appear to exist in man or the mouse, suggesting that these two loci are on different chromosomes, or at least widely separated on the same chromosome. Presumably, a translocation of the duplicated gene occurred either at or after the time of duplication.

Another system which probably represents a series of duplications is that of the mammalian immunoglobulins. The three major immunoglobulins, the γ_2(γG), γ_1A(γA) and γ_1M(γM) globulins are thought to be composed of two different types of polypeptide chains (Fig. 12). One, the L-(light) chain, is common to all while the other, the H-(heavy) chain, is specific for each globulin. However, the fact that the various H-chains are similar in size and serve very similar functions suggests that they may have been derived from a common precursor, and the recent finding of Herzenberg (1964) that the loci for the mouse H-chain antigens of γ_1A(γA) and γ_2(γG) globulins are closely linked strongly favors this interpretation.

Another area in which duplications are being sought involves various

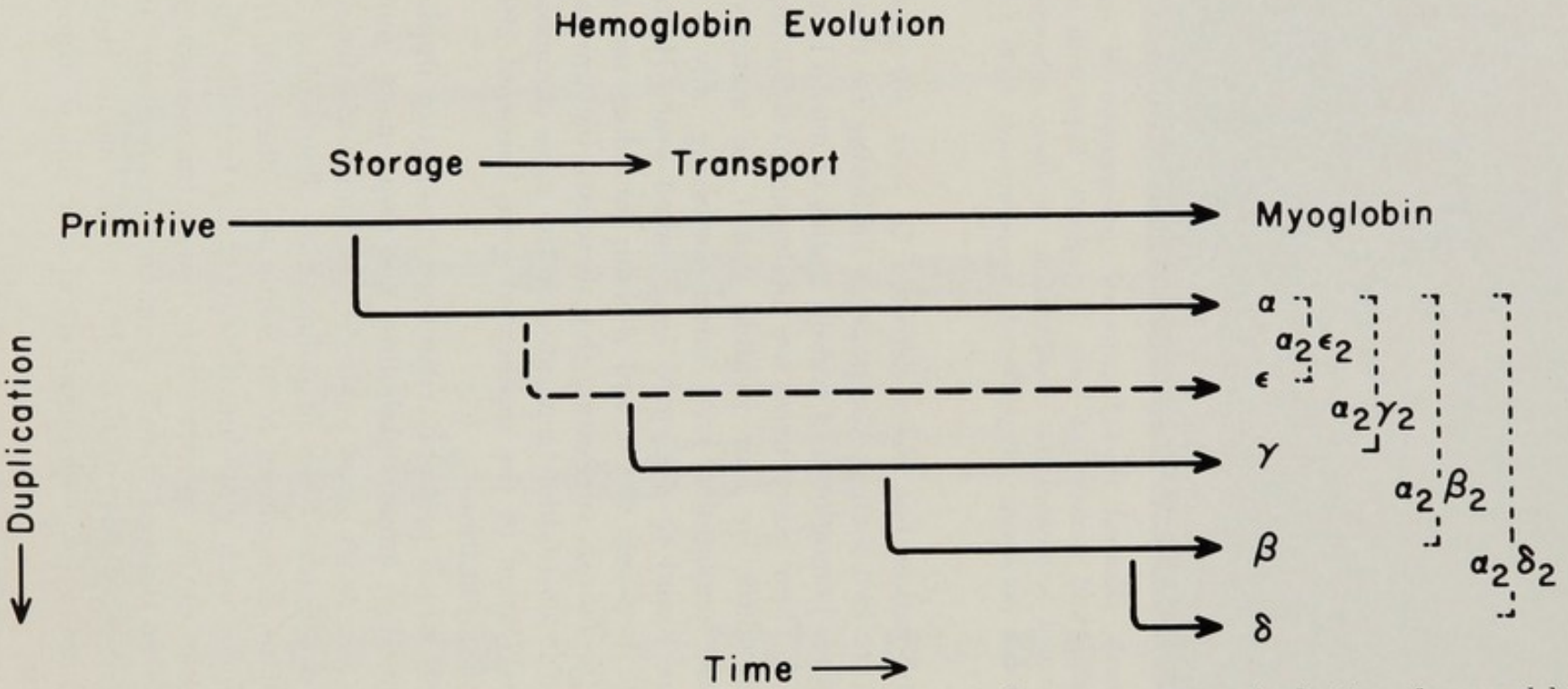


FIG. 10—Evolution of hemoglobin (modified from Ingram, 1961). Each vertical step represents a duplication of a myoglobin/hemoglobin locus while the horizontal axis represents evolutionary changes after duplication. Because of its partial similarity to the γ-chain, the origin of the ϵ-chain has been inserted between that of the α and γ-chains.

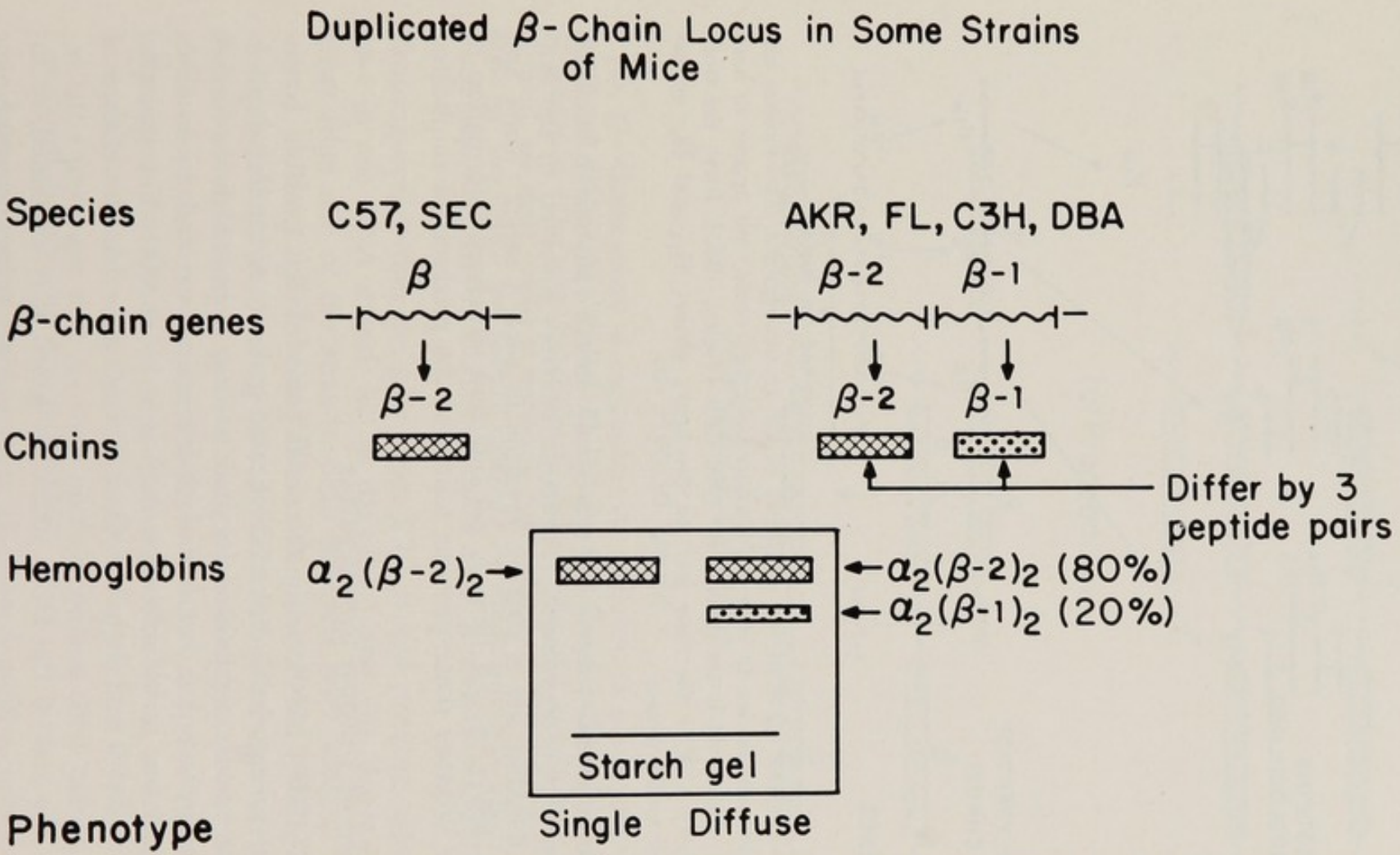


FIG. 11—A duplicated hemoglobin β-chain locus in mice. Similar to the linked human hemoglobin β and δ-chain genes, there are two linked β-chain genes in the AKR, FL, C3H, and DBA species of mice (Hutton et al., 1962a, b). The two chains in the mice are produced in a ratio of 4:1 while those in the human (β and δ) are in a ratio of about 50:1. Since lower primates lack the δ-chain found in man (Kunkel et al., 1957), it appears that the β-chain duplication in mice may represent an event independent of that giving rise to the δ-chain in higher primates.

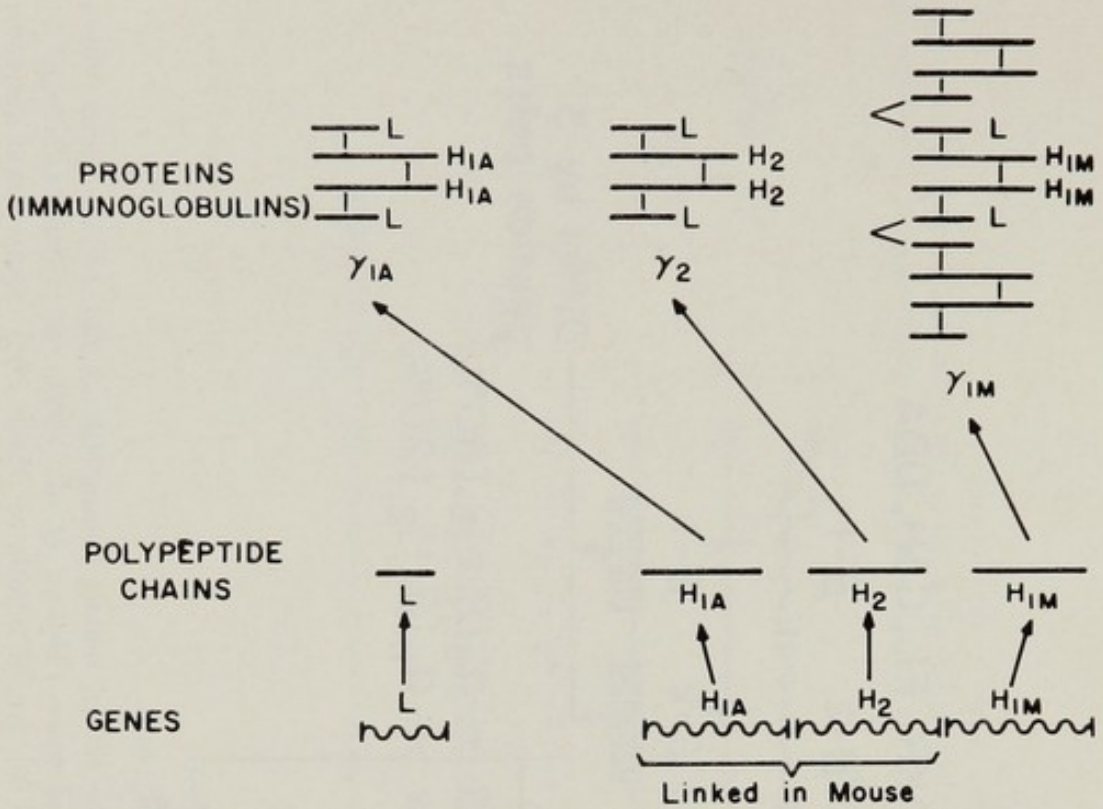


FIG. 12—Current views of the structures of the immunoglobins. The various immunoglobulins are known to share a common L (light) chain, but appear to have H (heavy) chains which are specific for each class (Fahey, 1963). From the work of Herzenberg (1964) the gene loci for the heavy chains, H_{1A} and H_{2}, appear to be linked in the mouse.

series of functionally related and genetically linked enzymes in bacteria. The group of ten coordinately regulated enzymes involved in the synthesis of histidine are a striking example of such a series (Ames and Hartman, 1963). As yet there is not sufficient structural information to determine whether these proteins represent the products of duplicated genes.

Propagation of Unequal Crossing-Over

Nance (1963) has recently discussed some of the possible consequences of having closely linked duplicated genes (i.e., tandem duplications). The various possibilities for gene pairings appear to favor further duplication by unequal, but *homologous* crossing-over and, in an autocatalytic manner, one duplication may lead to another. For example, with the linked β and δ-chain loci, a β-chain gene has the chance of correctly pairing with another β-chain gene or, if it can escape the influence of the rest of the DNA strand in which it is contained, of incorrectly pairing with a δ gene. If a β-δ-pairing occurs, crossing-over outside the pair (Fig. 13a) could produce duplication of one of these genes in one strand and a deletion of the same gene from the other.

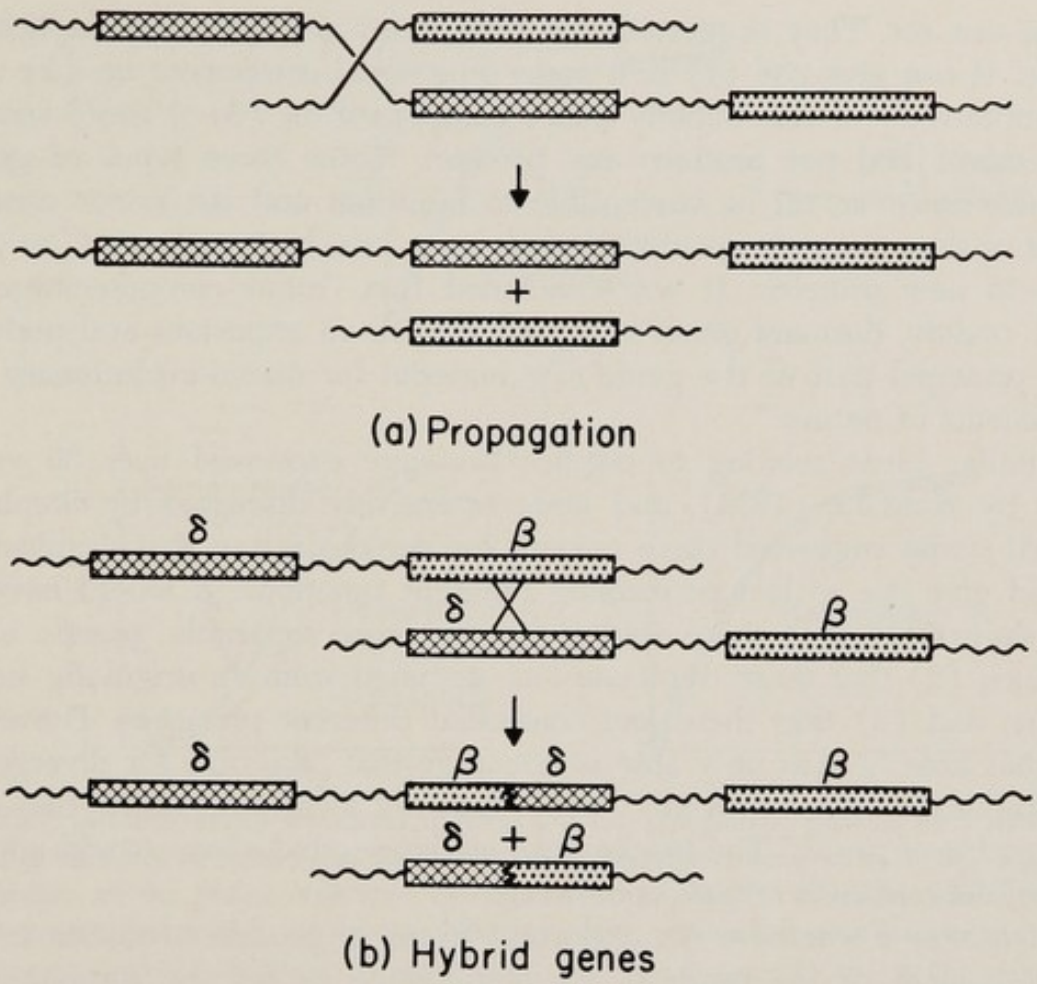


FIG. 13—Consequences of duplication: (a) formation of gene triplication in one strand and deletion in the other by homologous but unequal crossing over outside of gene loci. (The term "homologous" indicates that the paired genes, while not identical, are quite similar in nucleotide base sequence.) (b) Formation of hybrid genes by homologous but unequal crossing-over within gene loci. The δ-β type of fusion gene, with deletion of normal β and δ genes, is found in hemoglobin Lepore.

Crossing-over within the pair (Fig. 13b) would produce hybrid genes (β-δ in one DNA strand, δ-β in the other) and also deletion of both genes from one of the strands. Such products have actually been observed in the Lepore hemoglobins (Baglioni, 1962, 1963). This type of hemoglobin was found to have a δ-like amino-terminal portion and a β-like carboxyl terminal portion. Patients homozygous for Lepore hemoglobin do not have any normal β or δ-chains and thus appear to be homozygous for the DNA strands from which the β and δ genes had been deleted and in which only the hybrid gene persisted.

Functional Significance of Duplications

Zuckerkandl and Pauling (1962) recently pointed out that the great utility of the process of duplication is that it allows for the preservation of a particular function while generating a new locus on which muta-

tions can act. They suggested that three fates are open to a duplicated gene. It can give rise (1) to a major functional component or, (2) to a quantitatively or functionally minor component, or (3) it could remain "dormant" and not produce any product. These three types of genes would, however, all be susceptible to mutation and the minor component or dormant genes could, at some later date, be "reactivated" to give rise to new proteins. It was concluded that "minor-component genes and, mainly, dormant genes may thus furnish an important and perhaps the principal part of the genic raw material for macro-evolutionary experiments of nature."

Similar ideas relating to duplication were expressed over 30 years ago by Haldane (1932), and were extensively discussed by Stephens (1951) who suggested three criteria for demonstrating that duplication could give rise to loci performing different functions: it would have to be shown (1) that there existed two or more separable genetic units or loci; (2) that these duplicate loci stemmed from an originally single locus; and (3) that these loci controlled different processes. However, at that time he was only able to conclude that "the case for divergence of duplicates is not proved . . . this does not mean necessarily that it is incapable of proof." The biochemical evidence cited above would appear to fulfill Stephens's criteria completely.

Multimer Formation. An important event in protein evolution is the development of the property for aggregation or for the formation of *multimers.* (The term "multimer," as opposed to "polymer," has recently been introduced by Crick and Orgel (1964) to designate protein subunit aggregates of definite composition and quaternary structure which are not necessarily linear and do not possess covalent links.) For example, myoglobin exists only as a monomer, but the β and γ hemoglobin chains which appear to be derived from a myoglobin-like precursor, are able to form tetramers (β_4 = hemoglobin H; γ_4 = hemoglobin Barts').

Several years ago, Wald (1952) pointed out that hemoglobin probably passed through three stages in its evolution: (1) heme enzymes of cellular respiration (such as cytochrome oxidase), (2) cell and tissue hemoglobins (such as myoglobin) for oxygen *storage,* and (3) the circulatory hemoglobins for oxygen *transport.* While there is little evidence to support the notion of the evolution of hemoglobin from cytochrome, the transition from a myoglobin-like protein to hemoglobin appears to be valid. Four biochemical "innovations" are associated with this transition: (1) heme-heme interaction, as manifested in the sigmoid-shaped oxygen-binding curve, (2) a decrease in oxygen affinity (the shift of the binding

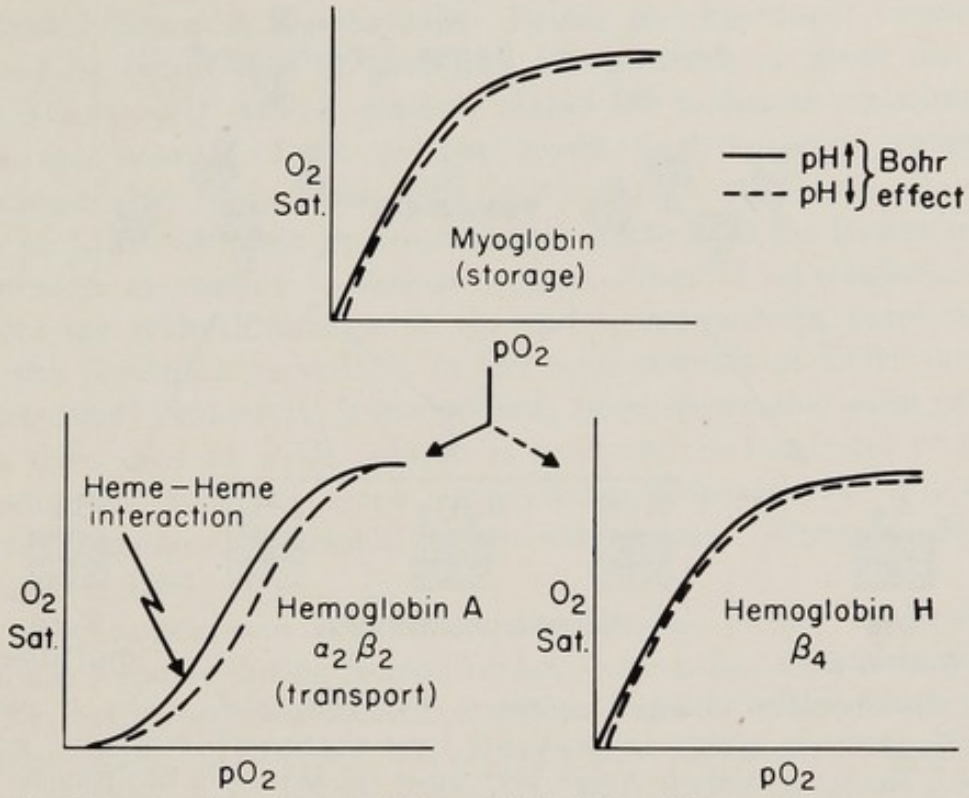


FIG. 14—Functional evolution of hemoglobin. Myoglobin, existing as a *monomer*, has a high oxgen affinity, no Bohr effect, and no heme-heme interaction. With the evolution of the *mixed multimer*, hemoglobin A ($\alpha_2^A\beta_2^A$), the Bohr effect (different oxygen saturation curves at different pH's), heme-heme interaction (sigmoid shape of curve), and lower oxygen affinity (shift of curve to the right) appeared. However, the *pure-multimer* by-product, hemoglobin H (β^4), has none of the properties of hemoglobin A, and neither hemoglobin H nor myoglobin bind haptoglobin.

curve to the right), (3) the Bohr effect (the relationship between pH and oxygen binding capacity) (Benesch and Benesch, 1963; Manwell, 1958) (Fig. 14), and (4) the property of binding to haptoglobin (Nagel and Ranney, 1964). The β and γ hemoglobin chains can form homogeneous tetramers, but neither these tetramers, nor the monomers of the various chains, nor myoglobin itself, have any of the biochemical attributes of circulating hemoglobin (Benesch and Benesch, 1964). These desirable functional properties appeared only when the β and γ-chains became capable of combining with α-chains, thereby forming the characteristic mixed hemoglobin multimers $\alpha_2\beta_2$ and $\alpha_2\gamma_2$. In the case of hemoglobin, therefore, both duplication and multimerization, which together allowed for the formation of mixed multimers, were required for the evolution of the efficient oxygen-carrying hemoglobin of higher organisms.

A large element of functional subtlety may also be introduced by the

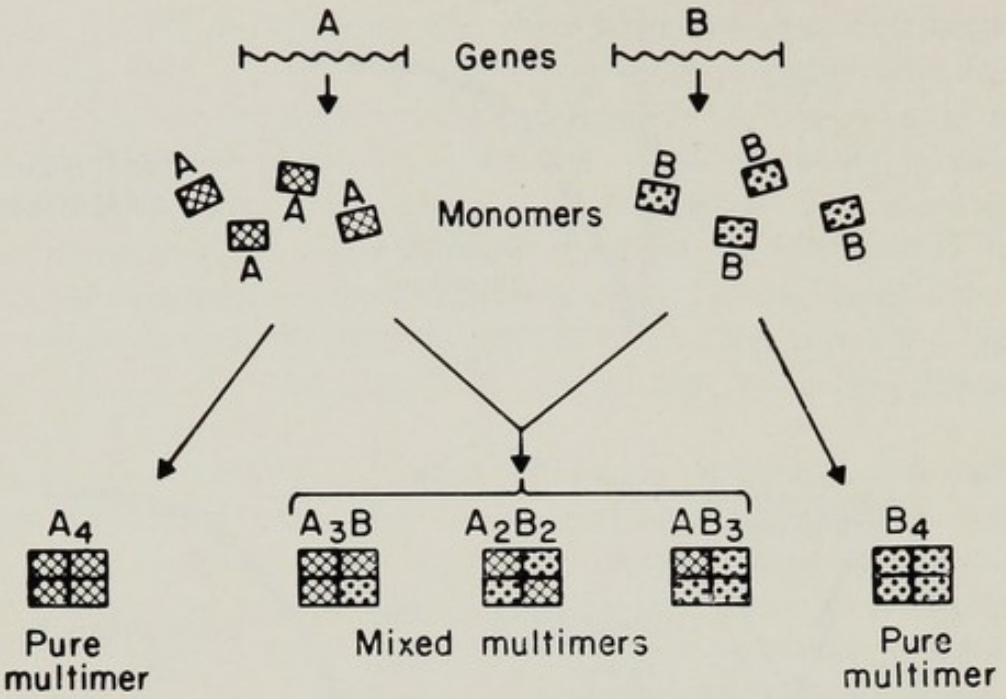


FIG. 15—Formation of mixed multimers. Two polypeptide chains, if allowed to aggregate randomly, will given rise to five types of tetramer; two "pure" and three "mixed." The lactate dehydrogenase isoenzymes are formed in this manner.

formation of mixed multimers. This is well illustrated by the enzyme, lactate dehydrogenase (LDH). Two isoenzymes of LDH, 1 and 5, represent "pure" tetramers of A or B subunits, and the available chemical evidence is compatible with the hypothesis of a common origin for these subunits (Markert, 1963). This possibility was first suggested by Vesell and Bearn (1962) who also noted that the red cells of snakes and frogs had only one LDH band on starch gel electrophoresis. If this is true for other tissues of these animals, it might indicate that duplication of the LDH locus occurred after the divergence of amphibia and reptiles from the main vertebrate evolutionary pathway. In bacteria (*B. subtilis*) only one type of LDH is found (Yoshida, 1965).

When the subunits of LDH are combined in various proportions, a series of mixed tetramers (isoenzymes 2, 3, and 4) is produced (Fig. 15). These mixed tetramers possess a range of properties (such as their inhibition by various agents) intermediate between those of the pure tetramers (Dawson, Goodfriend and Kaplan, 1964). Thus, a whole set of related, but not identical, enzymes has been generated.

A shift in equilibrium between monomer and pure multimer may sometimes have similar effects. For example, the activity of glutamic dehydrogenase, when it exists as a monomer, is greater with alanine than with glutamate as substrate, while the reverse is true when the enzyme exists as a (pure) multimer (Tomkins et al., 1963).

Duplicated Genes in Development. Beside the functional versatility introduced by duplication of gene loci, the presence of genes for two or more functionally related proteins allows for temporal variations in the type and amount of the proteins made. Such variation seems to occur primarily in conjunction with developmental processes. Thus, in the case of LDH, different proportions and amounts of the two subunits are produced in various tissues at various times of development, so that there are wide differences in the isoenzyme patterns (and therefore in the function) as well as in the total amount of LDH present (Markert, 1964). Moreover, it has recently been shown that after pubescence a third type of LDH subunit is produced in the testes of man, other mammals, and the pigeon. It has been proposed that this third locus may somehow be related to spermatogenesis (Zinkham, Blanco and Kupshyk, 1964).

The early appearance and subsequent decline in the synthesis of hemoglobin γ-chains during human embryogenesis has long been recognized. As was already mentioned, a second human fetal or embryonic hemoglobin chain, the ϵ-chain, has recently been discovered (Huehns et al., 1964a). This chain, which appears to have a few peptides in common with the β and γ-chains, is the unique hemoglobin chain very early in embryonic life, appearing even *before* the α-chain (Hecht et al., unpublished data). It gives rise to two different hemoglobins: Gower-1 (ϵ_4) and Gower-2 ($\alpha_2\epsilon_2$). By the time fetuses are 8.5 cm. in length (and probably much earlier) ϵ-chain synthesis has ceased completely. In individuals with the D_1 trisomy syndrome, there is a delay in the rates at which γ and ϵ-chain synthesis decline (Huehns et al., 1964b), suggesting that some connection between the mechanisms controlling the synthesis of these two chains may exist. However, the fact that the Gower hemoglobins disappear much more rapidly than does fetal hemoglobin indicates that these mechanisms are not identical for the two chains. Thus, there appears to be a sequential order to the onset and cessation of synthesis of the non-α hemoglobin chains, namely $\epsilon \rightarrow \gamma \rightarrow \beta$ and δ.

Ontogeny and Phylogeny

The sequential appearance of proteins during development raises the question of whether we are observing, in a system such as that of the hemoglobins, an example of "recapitulation." This concept, commonly, though erroneously, stated as "ontogeny recapitulates phylogeny," has had a long and ill-fated history (de Beer, 1958), but some of von Baer's original premises remain unchallenged. In particular, it is quite clear

that developmental stages during the ontogeny of a lower species may be repeated during the ontogeny of a higher one, even though there have been changes, sometimes quite great, in their functional significance. However, the preservation of a "phylogenetic vestige," whether because it subserves some necessary function or because it has, for one reason or another, not been eliminated (e.g., because of its location in some essential group of genes, or "supergene"), does not insure that the time of its ontogenetic expression is related to the time of its phylogenetic appearance. Thus, while it appears quite likely that the hemoglobin γ-chain, which ontologically precedes the β-chain, also preceded the β-chain phylogenetically (see below), the same need not be true with relation to the ϵ-chain. Therefore, while it is tempting to suggest that the ϵ-chain represents the earliest non-α-chain, and that the γ and β-chains developed later, there is at present no evidence for this. Embryonic or larval hemoglobins have been found in all major groups of vertebrates, and many lower species, such as the frog and the lamprey, have been found to have two or more unique larval components (Gratzer and Allison, 1960; Baglioni and Sparks, 1962; Manwell et al., 1963). Therefore, in order to completely trace the phylogenetic evolution of the several human hemoglobin chains, the amino acid sequences and developmental histories of chains from all taxonomic levels of vertebrates and invertebrates may be required, and even then the task may be difficult.

Another possible example of an ontogenetic-phylogenetic relationship is provided by the human immunoglobulins. Early in life, or after stimulation with a new antigen, the first antibodies that an individual makes are generally of the γM (19S macroglobulin type; later, the γG (7S) globulins appear (Smith and Eitzman, 1964). Similarly, in the lowest vertebrates found to have antibodies, the lampreys, paddlefishes, and gars, these antibodies appear to be localized in macroglobulins (Ashbach et al., 1964).

"Dispensible" Proteins

Occasionally, the function of a protein may no longer be required in a given situation or species so that the corresponding gene(s) is eliminated or "switched-off." A striking example of this is the absence of hemoglobin (and of red cells) in fishes of the family Chaenichthydae, the ice fish, and in the larvae of the eel Anguilla anguilla (Lehmann and Hunstman, 1961). The former, living in Antarctic waters at temperatures below 2° C., are able to function satisfactorily, albeit not actively, without red cells and hemoglobin.

Taxonomic Schemes and Evolutionary Time Scales

It has long been realized that the analysis of homologous proteins from different species would be useful in deducing the course of species evolution and checking established taxonomic relationships. In 1904, there appeared Nuttall's treatise on "Blood Immunity and Blood Relationship, a demonstration of certain blood relationships amongst animals by means of the precipitin test for blood." On the basis of 16,000 precipitin tests on serum and egg proteins, Nuttall was able to show antigenic relationships among proteins from closely related species, and actually attempted to quantitate the degree of relationship and to apply the results to problems of taxonomy. He concluded, for example, that Darwin was correct in assuming "that the Old World apes are more closely allied to man than are the New World apes." Modern immunochemists have examined many diverse proteins (hemoglobin, specific serum components, lens, thyroglobulin and LDH) and have found, with relatively few exceptions, that the closer the species (on conventional taxonomic grounds) the greater the cross-reactivity among their proteins (Goodman, 1962; Halbert and Manski, 1963; Shim and Bearn, 1964; Williams, 1964). Immunological reactions are dependent on the surface properties of the antigen and even small changes in amino acid sequence can seriously affect the surface (see below). Therefore, while antigenic cross-reactivity is probably good evidence for the relatedness of proteins, related proteins need not necessarily react similarly in immunochemical assays. As a result, the analysis of sequences is emerging as a more powerful tool for investigating the fine details of protein evolution.

Doolittle and Blombäck (1964) have recently examined a series of fibrinogen peptides obtained from several species of artiodactyls (hoofed mammals). By proper ordering of the peptides, it is possible to obtain a progression involving a minimum of amino acid substitutions. If it is postulated that the closeness of two species to some common ancestor is proportional to the similarity of the sequences of their proteins, the evolutionary relationships shown in Figure 16 are obtained. In general, the relationships observed among the fibrinopeptides (of which only a segment is shown in the figure) follows the classic taxonomic scheme. However, there is one major discrepancy: the sheep and goat peptides (which are identical) differ by only one residue from the reindeer peptide, but both groups differ by four residues from the ox peptide. This would suggest that sheep, goats, and reindeer had a common ancestor less distant (in evolutionary terms) from each of them than was the ancestor common to sheep, goats, and oxen. However, on morphologic

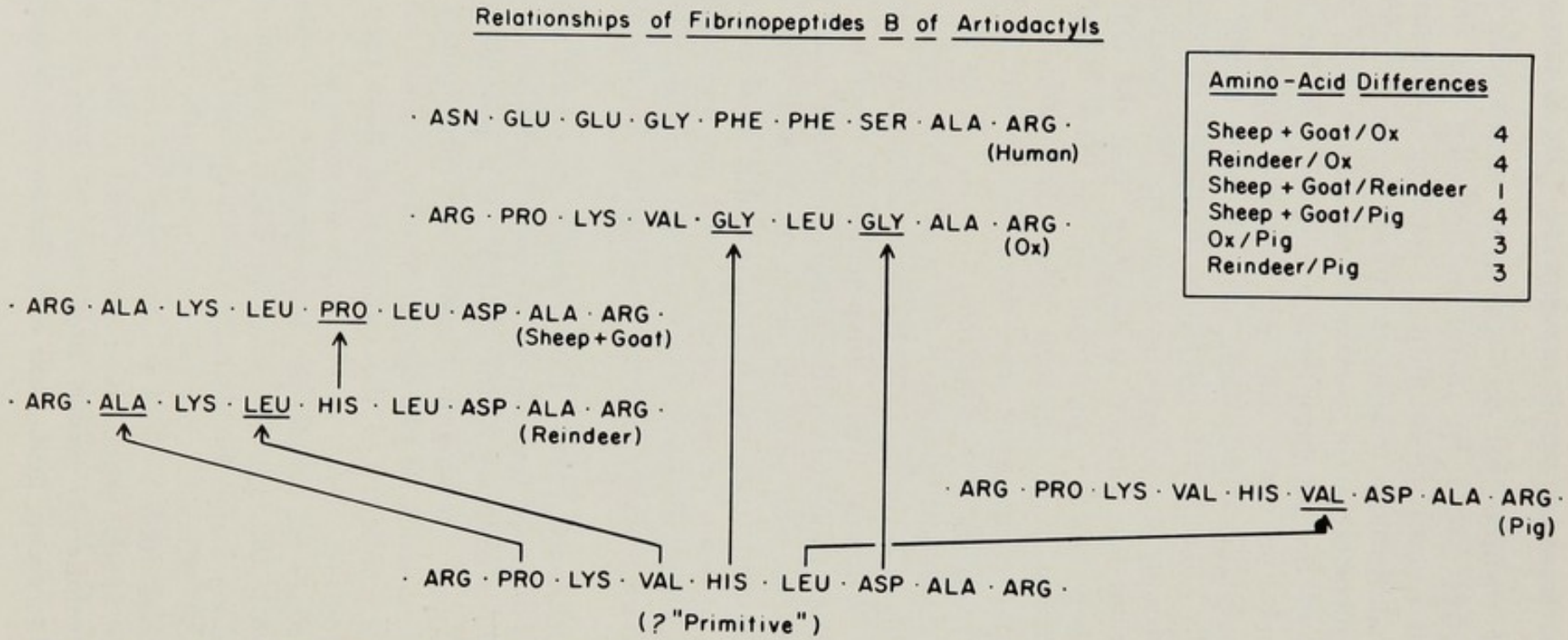


FIG. 16—Postulated scheme for the evolution of fibrinopeptides of hoofed mammals. This scheme, derived from an analysis of a small peptide segment from fibrinopeptide B (Doolittle and Blombäck, 1964) illustrates the method of deducing taxonomic relationships from amino-acid sequence comparisons. The scheme shown here differs from the classic taxonomic scheme in that, in the latter, sheep and goats are supposedly more closely related to the ox than to the reindeer. The human peptide is shown for comparison.

grounds, the latter has been postulated to represent the closer relationship. Which of the two schemes will ultimately prove to be correct remains to be seen, and the result will give some indication of the degree of reliance that can be placed on this type of biochemical evidence.

Another example of this approach is provided by the data of Hill and co-workers (1963) on the primate hemoglobin β-chains. These workers showed the β-chains of the lower primates closely resemble the γ-chains of man. During primate evolution there has been a gradual transition to the human β-like chains found in the higher primates (Fig. 17). Very few residues are found which are represented in neither the human β or γ-chains. If isoleucine content is taken as a mark of γ-like character (β-chains have none), a similar trend is seen from the analysis of whole hemoglobin amino acid compositions by Riggs (1963): isoleucine is very high in the hemoglobins (and presumably in the β-chains) of mouse and guinea pig, but is totally absent in those of horse and man. It is also of interest that adult hemoglobin from prosimians is resistant to alkaline denaturation, a property characteristic of human fetal hemoglobin. These findings are of great interest since, as has already been mentioned, it has been postulated that the human β-chain did, indeed, evolve from a γ-like precursor. (The conceptual basis for the study of evolutionary relationships by comparison of the amino acid sequences of a given protein in different species is not entirely new. A similar approach (termed "glottochronology") has been usefully employed in linguistic studies of the evolution of words in different, but related languages (Lees, 1953).)

In their review of protein evolution, Zuckerkandl and Pauling (1962) estimated the rate of mutation by a comparison of protein sequences in different species, using known paleontological information as an index of the time scale. Utilizing the human and horse α-chains as their standard (17 amino acid differences) and assuming that each of these α-chains had undergone about 9 random mutations and that the two species diverged 130 million years ago, they calculated a figure of approximately 14.5 million years per mutation per 150 amino-acid residues, or 22 million years per mutation per 100 residues (Table 1). With this figure, they then estimated the time since the divergence of the various human hemoglobin chains from one another. Margoliash (1963) has carried out similar calculations for the evolution of cytochrome *c*, and when the method Zuckerkandl and Pauling is used, a rate of approximately 22 million years per mutation per 100 amino acids is again obtained. Doolittle and Blombäck (1964), in following the amino acid exchanges at a single site in fibrinopeptide A, estimated a rate of 10 million years per

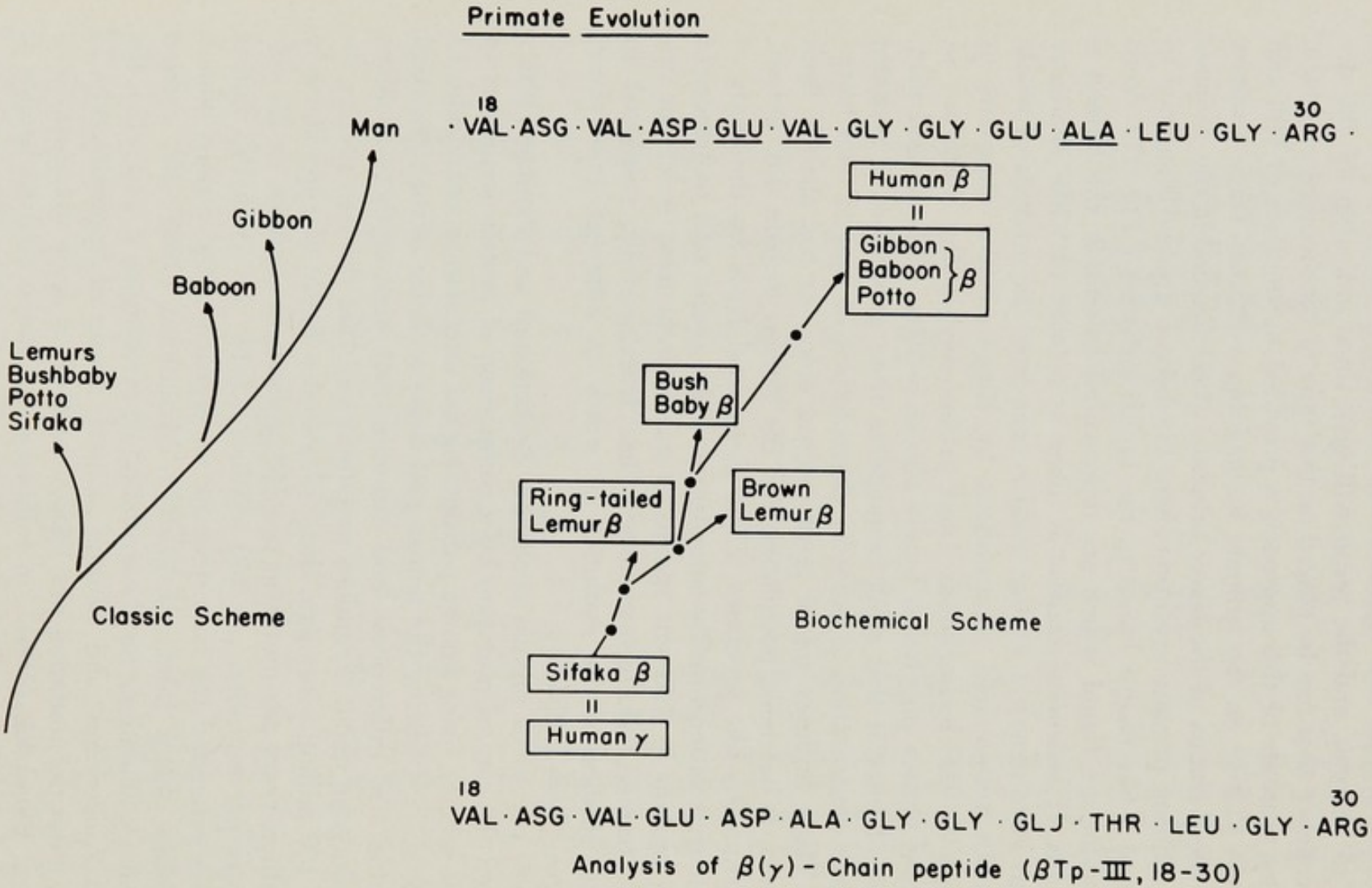


FIG. 17—Evolution of primates as reflected in a single hemoglobin β-chain peptide from several species (after Hill et al., 1963). Each "joint" in the diagram on the right represents a single amino-acid substitution. It should be noted that the β-chain peptides of lower primates closely resemble the homologous human γ-chain peptide, and this relationship appears to be true for the whole β-chain.

TABLE 1.—*Estimates of Rate of Protein Mutation (Single Amino Acid Substitutions)*

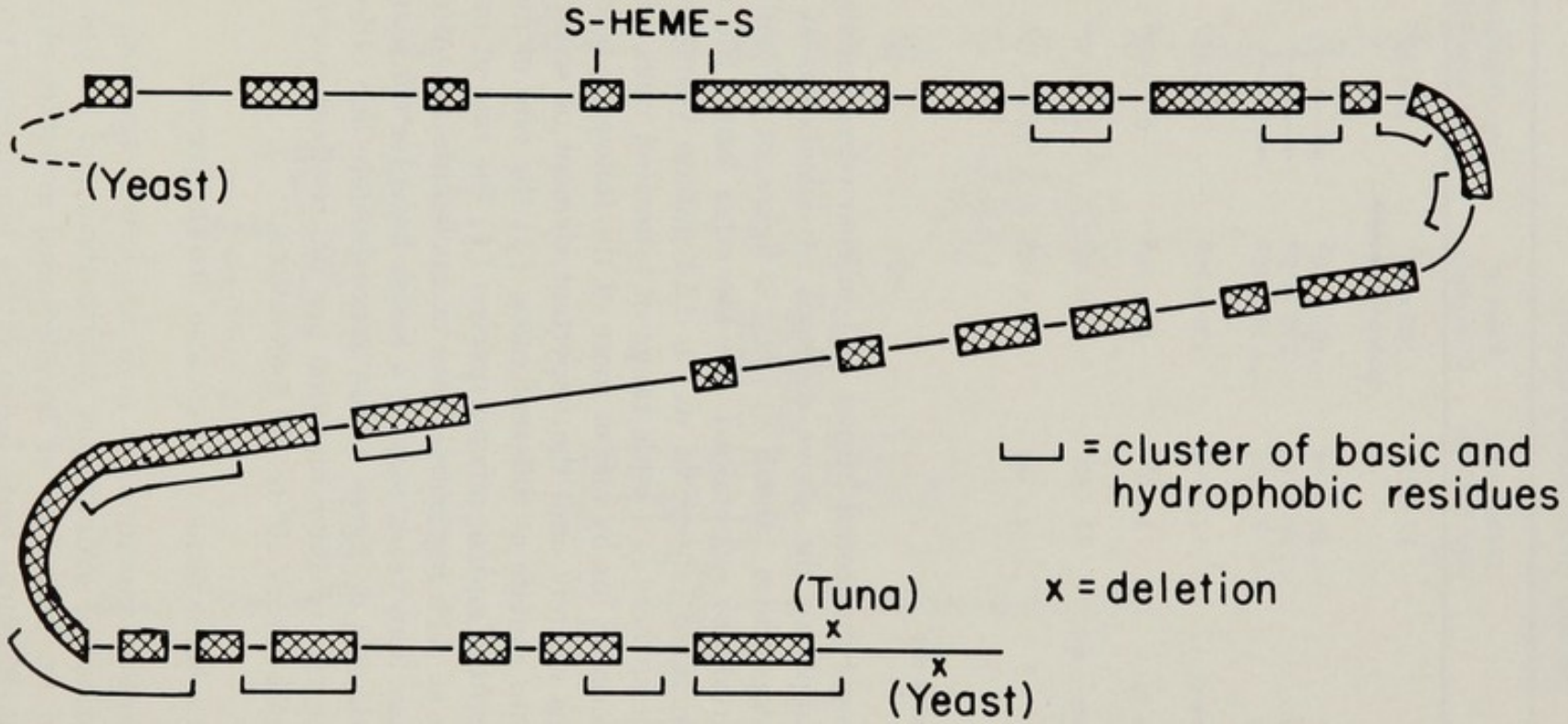
Protein and Species	Differences/ Total Residues	Years of Divergence	Years/Mutation/ 100 Amino Acids
Hemoglobin: α-chain (horse / human)	17 / 141	130×10^6 (paleontological)	22×10^6
Hemoglobin: α / γ (human)	~88 / 146	$\sim 635 \times 10^6$	
β / γ (human)	~40 / 146	$\sim 290 \times 10^6$	
β / δ (human)	~ 8 / 146	$\sim 58 \times 10^6$	
Cytochrome C (horse / human)	12 / 104	130×10^6	22×10^6
[Fibrinopeptide A, residue 13	4 / 1*	40×10^6	0.1×10^6]
Fibrinopeptides A & B (sheep / ox)	17 / 40	15×10^6	0.7×10^6
Ribonuclease (sheep / ox)	3 / 124	15×10^6	12.4×10^6

*) 4 different residues at same site

mutation within a single nucleotide triplet (0.1 million years/mutation/100 residues), but applying the above methods of calculation to the total A and B fibrinopeptides (sheep vs. ox), a figure of 0.7 million years/mutation/100 residues is obtained. On the other hand, the rate for pancreatic ribonuclease (sheep vs. ox) is 12.4 million years/mutation/100 residues. Thus, there is a wide range of estimated rates which may be partially accounted for by one or more of the following factors: (1) these calculations entirely omit the important element of selection; (2) individual proteins mutate at different rates; (3) the rate of mutation is not constant within and/or among species; (4) the rate of mutation is not the same at each nucleotide base or nucleotide base-triplet; (5) generations, rather than years, may be a better basis for calculation. Nevertheless, the estimates do agree within approximately one order of magnitude, indicating one effective mutation per 100 residues every 10^6 to 10^7 years, or, roughly every 10^5 to 10^6 generations.

HOMOLOGOUS MUTATIONS IN MAN AND OTHER SPECIES

The finding of identical mutations in man and lower organisms may provide some indications of evolutionary relationships and possible selective factors. Proof for homology of an autosomal mutation requires demonstration of the identical basic lesion in the homologous protein of man and the infrahuman species. Linkage information is usually not



Areas of Identity in the Sequences of Cytochromes C
(From man, chicken, pig, horse, ox, tuna, and yeast)

FIG. 18—Sequence homologies in the cytochromes *c* from various species. The blocks, drawn approximately to scale, represent areas in which identical amino acids are found in the proteins from all of the species.

available since few autosomal genes have been mapped in mammals. Moreover, identical genes may have been translocated to different chromosomes in different species. Although a few animal conditions, such as hereditary spherocytosis in the deermouse (Anderson, Huestis and Motulsky, 1960) appear pathophysiologically very similar to human diseases, significant differences in phenotype usually exist between species and detailed biochemical studies have not yet been carried out for most mutations.

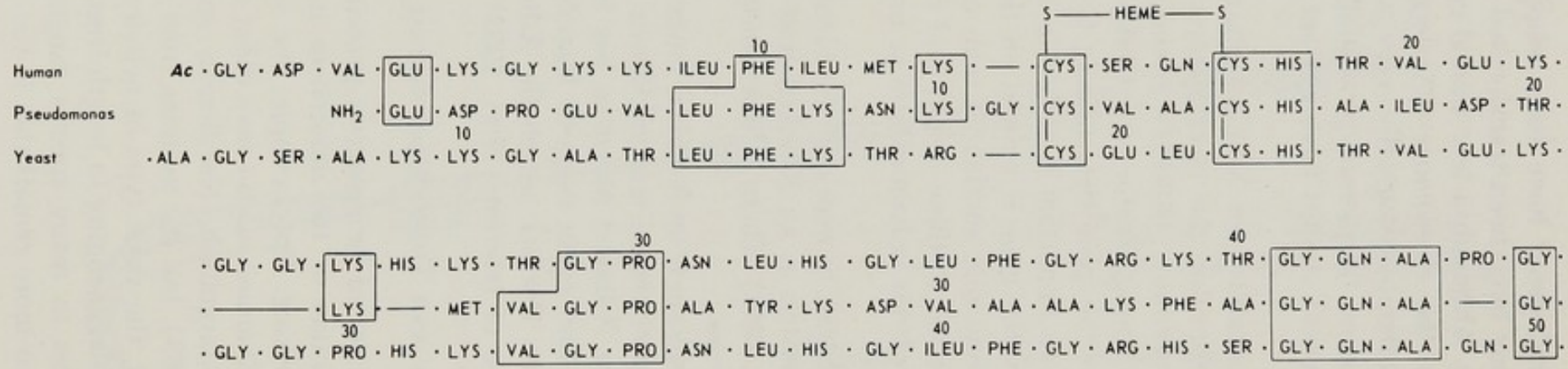
Homologies are easier to establish for X-linked mutations. The recent finding that the polymorphism for the electrophoretic phenotypes of glucose-6-phosphate dehydrogenase (G-6-PD) already exists as an X-linked trait in drosophila (Young, Porter and Childs, 1964) is of interest and may indicate that the gene determining G-6-PD (or a gene closely linked to it) serves an important function in sexual differentiation. This could explain why the gene is carried on the X chromosome in species as far removed from one another as man and fruitfly. However, chance could also have led to these results and definite proof will have to await the detection of this polymorphism as an X-linked trait in other species.

Hemophilia is an X-linked trait in man and the identical deficiency of antihemophilic globulin inherited as an X-linked trait has been discovered in dogs (Graham et al., 1949). There is little question that the same gene is affected in both species.

Anhidrotic ectodermal dysplasia is an X-linked mutation in man manifesting with hypodontia, hypotrichosis, and anhidrosis (Perabo, Velasco and Prader, 1956). A similar X-linked mutation has been described in cattle (Drieux et al., 1950) and again suggests homology between two unrelated mammalian species. Further search for X-linked homologous mutations may, therefore, be of interest (Hutt, 1953).

Amino Acid Sequence and Three Dimensional Structure

When one compares a series of homologous proteins obtained from different species, the magnitude of the differences in the amino acid sequences is often quite striking. For example, the sequence of cytochrome *c* (approximately 100 amino acids) may differ among species by as many as 40 residues (Margoliash, Needleman and Stewart, 1963; Kreil, 1963; Narita et al., 1963), but the proteins from all species retain, to a greater or lesser extent, the same type of activity. Moreover, over 50 residues, occurring in clusters ranging in length from 1 to 11 residues, are found in identical places in many species ranging from yeast to man (Fig. 18). Perhaps even more remarkable is the fact that, despite



AMINO-ACID SEQUENCE HOMOLOGIES AMONG CYTOCHROMES C
FROM MAN, PSEUDOMONAS, AND YEAST

FIG. 19—Homologous amino acid sequences from the cytochromes *c* of man, yeast, and *Pseudomonas*.

large areas of difference, the sequence of the first 39 residues of the cytochrome *c* from *Pseudomonas* (Ambler, 1963) is clearly homologous to the similar region from the yeast and human proteins (Fig. 19). Thus, in the case of the cytochromes *c*, evolutionary interrelationships between bacteria and man are clearly demonstrable.

Margoliash and co-workers (1963), in looking for evolutionary interrelationships among proteins of various species, pointed out that a second kind of clustering may also be present, one in which only basic (histidine, arginine, and lysine) and hydrophobic (valine, leucine, phenylalanine, etc.) residues are found (Fig. 18). Despite the fact that this latter type of clustering is somewhat variable, these authors suggest that it might represent some kind of structural or functional necessity. It is possible, however, that this observation concerning hydrophobic and basic residues may really be a restricted version of a more general rule. It is now widely agreed that the three-dimensional structure of a protein is primarily governed by its amino acid sequence (Anfinsen, 1962). Furthermore, it appears to be generally true, from both experimental and theoretical evidence, that hydrophobic (nonpolar) residues tend to be concentrated in the interiors of folded proteins, while hydrophilic (polar) residues are found on their surfaces (Kendrew, 1962; Richards, 1963). Therefore, it seems reasonable to suggest that the distribution of polar and hydrophobic residues along the polypeptide chain is of great importance in determining the final configuration of the protein molecule and, as a corollary, that alterations in this distribution could seriously affect the folding of the molecule (Fig. 20). In addition, there is evidence which indicates that the exact identity of many surface residues may not be of great importance for folding as long as their polar nature is not completely eliminated (Epstein and Goldberger, 1964). Considering that a highly specific three-dimensional structure is necessary for protein stability and function, the following "rule" can be set forth: if, during the evolution of a protein, similarity in structure and function is to be preserved, the amino acid exchanges (taken in the aggregate) should not significantly alter the distribution of polar and hydrophobic residues along the polypeptide chain (Epstein, Goldberger and Anfinsen, 1963).

On the basis of this formulation, the amino acid differences between proteins can be divided into three categories: (1) "permissible" exchanges (polar $\leftrightarrow$ polar, hydrophobic $\leftrightarrow$ hydrophobic); (2) "possibly permissible" exchanges (polar $\leftrightarrow$ glycine or alanine); (3) "nonpermissible" exchanges (polar $\leftrightarrow$ hydrophobic). If the differences between proteins are analyzed in this way, the results shown in Table 2 are ob-

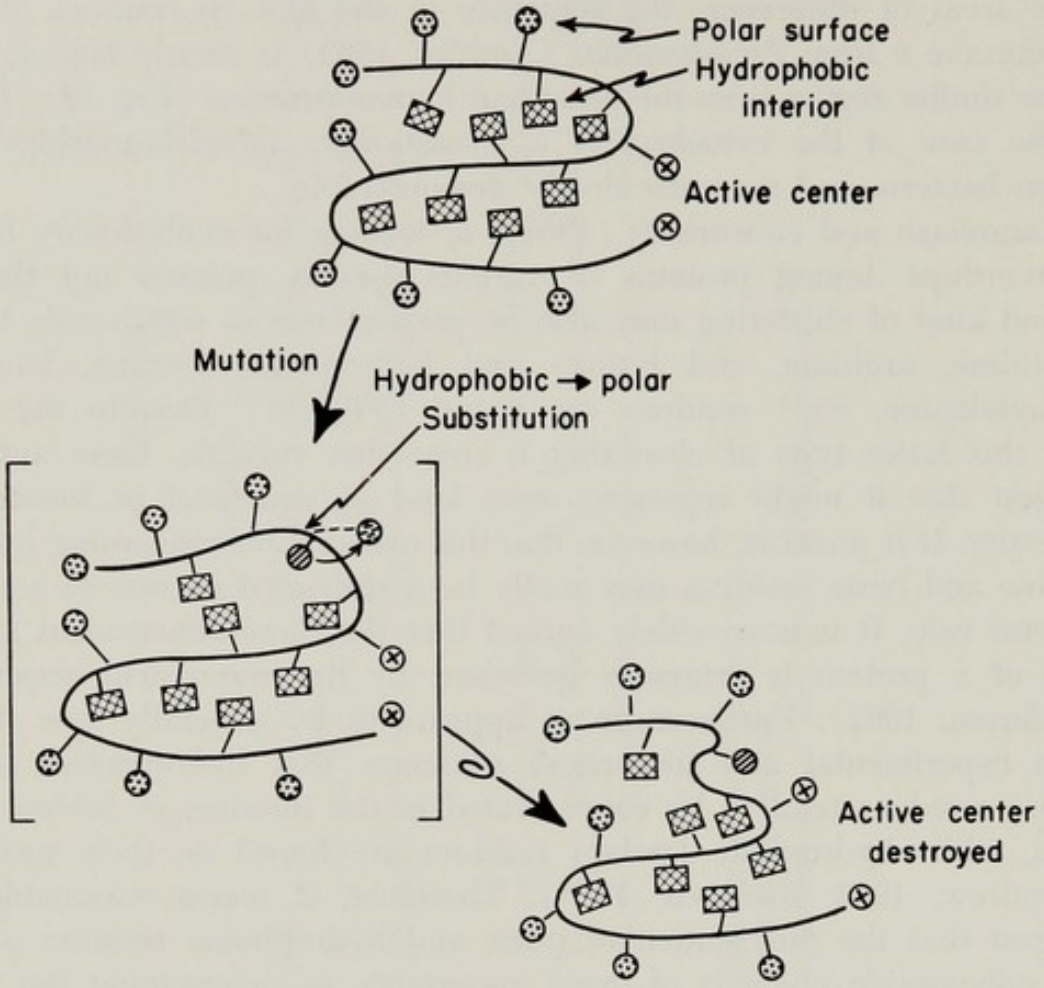


FIG. 20—Hypothetical effect on protein conformation of the substitution of a polar for a hydrophobic amino acid. The polar residue placed in the interior of the molecule by mutation attempts to attain a more favorable environment on the protein surface. As a result, the three-dimensional configuration of the molecule is altered, and there is a concomitant loss of the proper "active center" architecture and a decrease in overall stability.

tained (Epstein, 1964a). In most instances, "permissible" exchanges far outnumber "nonpermissible ones," the latter making up less than one-fourth of the total exchanges in the cytochrome *c* comparisons and less than one-eighth in the hemoglobin chain comparisons. In the hemoglobin comparisons these results appear to be quite significant since, as has already been mentioned, the structural and functional similarities of the various chains are well known. However, the fact that the proportion of nonpermissible exchanges can be as high as one-fourth suggests that these may be significant structural differences

TABLE 2—*Analysis of Observed Amino Acid Exchanges*

Proteins	Total Exchanges	TYPE I "Permissible" (Polar ⇄ Polar) (Non-Polar ⇄ Non-Polar)	TYPE II "Possibly Permissible" (Polar ⇄ Gly, Ala)	TYPE III "Non Permissible" (Polar ⇄ Non-Polar)
Cytochrome C				
Horse / Yeast	39	26	3	10 (26%)
Tuna / Yeast	41	29	7	5 (17%)
Hemoglobin				
Human: α / β	72	48	15	9 (13%)
Human: β / γ	40	24	12	4 (10%)
α: Human / Horse	17	9	6	2 (11%)
β: Human / Horse	21	13	6	2 (10%)

among proteins such as the cytochromes, and definite differences in activity have, in fact, been observed (Yamanaka and Okunuki, 1964).

Fisher (1964) has recently calculated the effect of chain length and the content of hydrophobic and polar residues on tertiary (three-dimensional) structure, and concluded that these factors are of great importance. He suggested, furthermore, that an increase in the proportion of hydrophobic residues can produce the transition from monomer to multimer, and such a change, although only of small magnitude, seems to have occurred in the transition from myoglobin to hemoglobin.

PRESENT DAY EVOLUTION

Implicit in the foregoing discussion have been several assumptions: (1) with few exceptions (i.e., propagation of duplicated genes), the mutational processes are essentially random; (2) mutations occur continually; and (3) selection operates to determine the nature of proteins which ultimately exist in any particular species. Mutations are the substrate for evolution and, as such, are constantly being acted upon by the forces of selection. The proteins which do persist presumably do so because they confer an advantage on the host. The consequences of mutation and selection will be considered separately.

Effects of Structural Gene Mutations on Proteins

Some of the wide range of molecular manifestations resulting from structural gene mutations are listed in Table 3. There is undoubtedly a large class of mutations which are phenotypically *silent* and are not de-

TABLE 3—*Effects of Structural Gene Mutations on Proteins and Their Synthesis*

1. Silent (not detectable chemically or functionally)
2. Innocuous
   a. electrophoretic variant (hemoglobin variants)
   b. antigenic variant (γ-globulin, Gm groups)
3. Serious
   a. direct alteration of "active center" residue or region (hemoglobins: M's, Zurich, Seattle)
   b. interference with folding or change in surface characteristics
      1) change in stability (G-6-Pd's: Chicago-1, Oklahoma-1)
      2) indirect change in "active center"
      3) change in ability to aggregate (? hemoglobin Porto Alegre)
   c. decreased rate of synthesis (hemoglobin variants)
4. Severe
   a. inability to fold
   b. failure of synthesis

tectable by conventional means other than detailed amino acid sequence analysis of the protein. In such postulated mutants the amino acid substitution would be such as not to alter charge or interfere with folding, activity or synthesis of the protein (e.g., substitution of aspartic for glutamic acid on some "unimportant" part of the protein surface).

The next level of effect consists of *innocuous* alterations of some nonessential aspect of protein structure. These include the electrophoretic and antigenic variants resulting from alterations in surface architecture. Many apparently innocuous electrophoretic variants are known among the hemoglobins and transferrins (Giblett, 1962), and Boerma and Huisman (1964) claim that a change of only one amino acid in hemoglobin may be detectable immunologically.

Other mutations, because of alterations in folding or a change in some vital residue(s), may have more *serious* and pronounced effects on protein function and structure. The functional effects of such single residue alterations involving an "active center" region are well illustrated by the several hemoglobin mutations which result in methemoglobin formation. Most of these involve a change at either a histidine residue that normally interacts with the heme iron (e.g., M-Boston, M-Kanakakee, M-Saskatoon, Zurich) or at a residue which, by virtue of being close to the heme ring, now becomes able to interact with the heme iron or to distort the local architecture of the region (M-Milwaukee, Hb-Seattle)

(Baglioni, 1963; Motulsky, 1964a). On the other hand, there are a group of mutations which appear to primarily affect the folding of the molecule. The resulting alterations in conformation may become manifest as changes in stability and ability to aggregate, and may or may not be associated with changes in function or electrophoretic mobility. This range of possibilities is clearly illustrated by some of the rarer glucose-6-phosphate dehydrogenase (G-6-PD) variants, in which there are marked alterations in stability (e.g., G-6-PD Chicago–1 and Oklahoma–1) in addition to changes in kinetic properties, electrophoretic mobility, and rate of synthesis (Kirkman et al., 1964a, 1964b; Motulsky, 1964a). Similarly, the hemoglobin S mutation (β6 glutamic acid $\rightarrow$ valine) is another example of the effect of an amino acid substitution on folding, albeit of a localized variety. Under conditions of deoxygenation, this substitution appears to allow for a specific hydrophobic interaction of β-chain residues one (valine) and six (now valine), resulting in the "stacking" of the hemoglobin molecules and distortion of the red cell (Murayama, 1964).

More *severe* interference with the ability of a protein to fold properly may result in the apparent total absence of the protein. Such instances of "nonsynthesis" have sometimes been attributed to some defect in regulatory or controller type genes. However, such mutations are likely, in many instances, to represent either the synthesis of a nonfoldable polypeptide chain or the total absence of polypeptide chain synthesis, both resulting from structural gene mutations (Epstein, 1964b).

Structural gene mutations may also affect the rate of protein synthesis. Numerous examples of this are known for various bacterial enzymes (Ames and Hartman, 1963) and the same is probably true for many human protein mutations, particularly those in which there has been a change in some physical characteristic (such as electrophoretic mobility) unaccompanied by an alteration in specific activity (e.g., abnormal hemoglobins and probably some of the G-PD variants).

Selection

Selective factors ultimately determine whether a mutation will be preserved or eliminated. The α-chains of the mammalian hemoglobins provide some interesting examples of how selection may operate. Baglioni and Colombo (1964) have pointed out that the amino acid sequence alterations of the α-chains of various animal hemoglobins have been observed at sites different from those involved in the known human α-chain mutations. The human mutations are generally associated with a decrease in the rate of protein synthesis while the different animal hemo-

globin α-chains are produced at rates similar to those of β-chain synthesis. Thus, selection appears to have preserved only those alterations which were not associated with decreased rates of synthesis of α-chains. Therefore, diminished hemoglobin α-chain production may be presumed to lower, to some extent, biological fitness.

Hill and co-workers (1963) have noted that the evolution of the primate α-chain has proceeded at a slower rate than that of the β-chain, and, likewise, that the number of human α-chain mutations both in type and frequency appears to be less than of β-chain mutations (however, the difference in the number of types is small and may not be significant). It has been suggested that any alteration in the α-chain that would interfere with its ability to combine with the other chains, even if it did not significantly alter the other properties of the chain, would seriously affect the synthesis of all types of hemoglobins (Gower 2 = $\alpha_2\epsilon_2$; F = $\alpha_2\gamma_2$; A = $\alpha_2\beta_2$; A_2 = $\alpha_2\delta_2$) and might, therefore, result in inviable fetuses. Thus, in the case of the hemoglobin α-chains, selection appears to operate for such "molecular" properties as three-dimensional structure and rate of synthesis.

An excellent example of the selective potential resulting from mutation is provided by studies of the hemoglobins of Dorset sheep (Naughton et al., 1963). These animals have two different β-chain alleles which produce chains differing slightly in their peptide maps and electrophoretic mobilities. Of greater importance, however, is the fact that the hemoglobin containing one of these β-chains has a greater affinity for oxygen than does the hemoglobin containing the other. It is quite conceivable that over the course of many thousands or millions of years the sheep living at high altitudes will eventually possess only (or predominantly) the high 0_2-affinity allele while those at low altitudes will have the other. If this occurred, the hemoglobin of the high altitude sheep would physiologically resemble that of the llama (Meshia, 1960), a species in which this type of selection may have already taken place.

Another more indirect selective mechanism affecting protein evolution is illustrated by the following example. The development of a placenta that allows the exchange of fetal and maternal plasma components may have slowed down the evolution of proteins (such as albumin) that are synthesized early in fetal life, but not of those, such as γ-globulin, made later (Goodman, 1962). A mutation leading to a protein antigenically different from that of the mother, could, if the protein crossed the placenta and if the differences were significant, induce maternal-fetal immunologic incompatibility. If the mutation involved an important protein and the antibody response were great enough, the offending offspring might be eliminated. Such a mutation, while in-

nocuous from the point of view of the protein, could thus be lethal for the host.

Selection and Human Proteins

When many human proteins have been carefully studied in different populations, a variety of mutations affecting their structures have been detected. Frequently, several common variants and many rare types are found (cf., hemoglobins and G-6-PD mutants). The frequency of the rarer types can generally be explained by mutation alone, but selection usually needs to be invoked to account for the more common polymorphisms.

In general, mutations which grossly interfere with function of a physiologically important enzyme or protein may have physiologic manifestations ranging from lethality in heterozygotes to producing harmful effects in homozygotes. Usually this type of mutation seriously lowers biologic fitness and tends to be more or less rapidly eliminated from the population. Protein alterations of the silent or innocuous types may be selectively quite neutral and raise difficult problems in population genetics. Studies of the last few years suggest that such alterations are common and that their relatively high frequencies are not accountable for by mutation alone. Although genetic drift in relatively small populations, if followed by rapid population expansion, may occasionally be an explanation, the ubiquity of the chemical polymorphisms renders this explanation unlikely. Subtle selection features depending on slight changes in activity or on unknown functions may possibly be operative in these systems. However, it is possible that some polymorphisms are now selectively neutral but reflect relics of selective forces such as infectious diseases or starvation which acted in the distant past (Motulsky, 1960).

Evidence for a still-existing selective advantage vis-a-vis an infecting agent is strong for hemoglobin S and the African and Mediterranean variants of G-6-PD deficiency. Carriers of these mutations have improved biologic fitness in an environment infested with falciparum malaria (Motulsky, 1964b). It is likely that a similar mechanism also explains the high frequency of β-thalassemia. However, very little is known about selective factors for most protein polymorphisms and much additional work in population genetics is necessary.

Conclusion

Many pathways of change are open to a primitive protein (Fig. 21). Some allow for preservation and improvement of function, while others

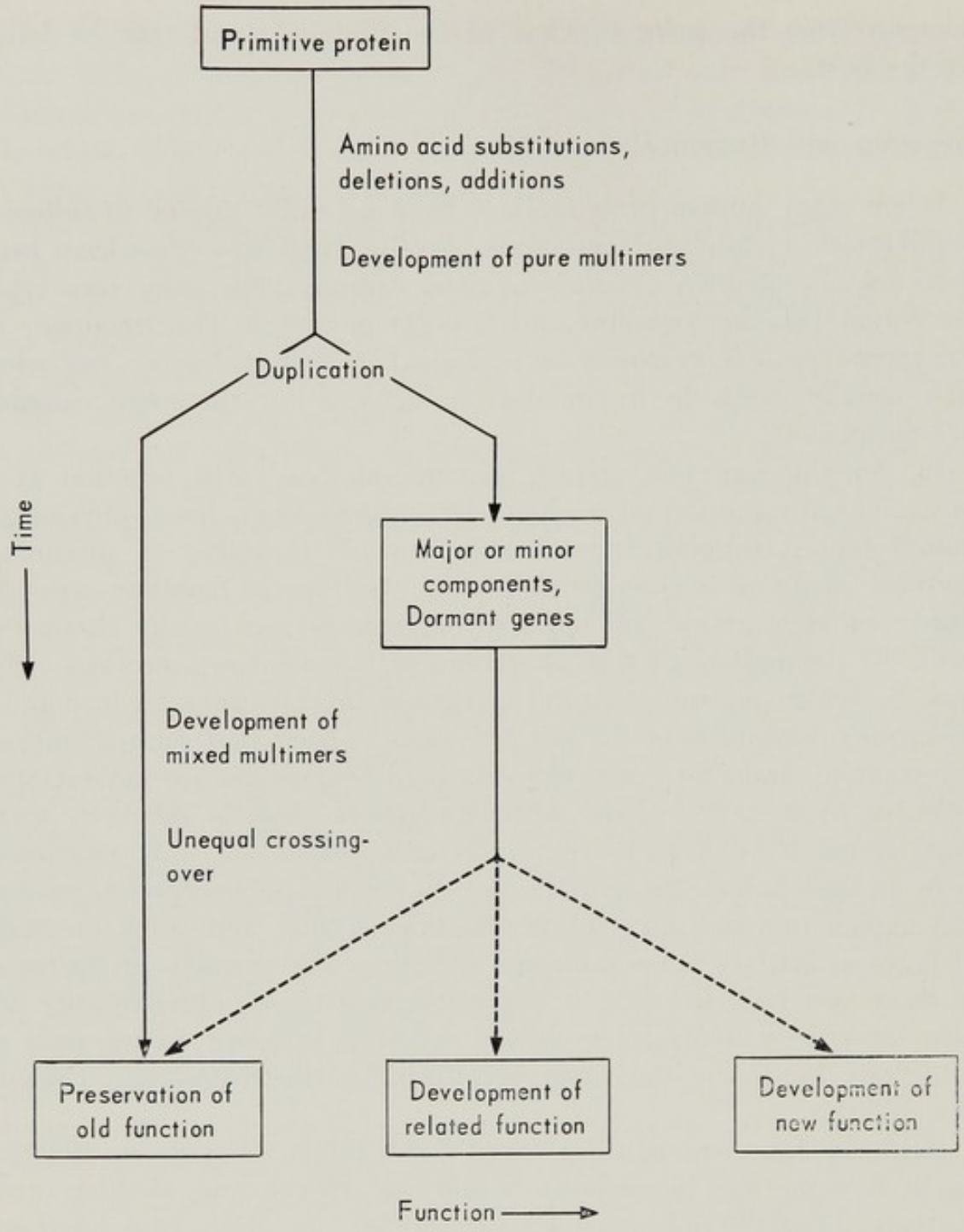


FIG. 21—General scheme of protein evolution.

give rise to new proteins with different functions. These processes depend on repeated mutational events of many types including: amino acid substitutions, additions, and deletions; partial or complete duplications; and the formation of hybrid proteins. The ultimate fate of the products of such mutations are controlled by the forces of selection

which determine whether a particular protein is to be preserved in or eliminated from the species.

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Chapter 5

Genetic Control of Isozyme Patterns in Human Tissues

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The term isozyme was coined by Markert and Møller in 1959 to refer to multiple molecular forms of an enzyme with similar substrate specificity. The word, which has enjoyed much popularity, and the concepts underlying it, which have been widely promulgated, served to focus attention on the heterogeneity of enzymes previously considered homogeneous and by so doing to create a new area of biochemical interest. The application of isozymes to diverse biological problems elevated the phenomenon of multiple molecular forms of enzymes into a general principle. Although in cases of particular isozyme systems plausible explanations have been advanced for this seemingly uneconomical situation, in other isozymic systems the reasons for the existence of multiple proteins all ostensibly performing a similar enzymatic function within the cell remain obscure. As isozymes were isolated and studied, it became clear that they differed significantly from one another in kinetic behavior toward a given substrate, as well as in substrate specificity. These observations required an extension and relaxation of the initial definition and today the term remains somewhat indefinite, although the chemistry of several isozymic systems has been elucidated.

In 1959 Markert and Møller cautioned against designating as isozymes obviously different enzymes that had broad, overlapping specificities and merely shared one or several substrates. The admonition still applies, but precise criteria of chemical structure or substrate specificity that must be fulfilled for two proteins to be considered isozymes have not been discussed recently. What has drawn considerable attention in the literature are the controversy over the numbering system for the lactic

dehydrogenase (LDH) isozymes (Wieme, 1962) and the issue of whether the word "isoenzyme" is preferable to "isozyme." Today some avoid the term isozyme entirely for fear of imprecision, whereas others apply it indiscriminately and thus diminish its significance by depriving it of the ideas and concepts it represents. The virtue of the term is that it emphasizes the existence within a single cell or tissue of a group of proteins similar in function and structure. This situation has now been described for so many different enzymes that it cannot be dismissed as an isolated, and therefore perhaps insignificant, event. As the generality of the principle became appreciated, isozymes were utilized in many different biological contexts, thereby arousing considerable interest and speculation.

The first biological application of multiple molecular forms of an enzyme antedated the term isozyme and depended on the observation that normal human serum exhibited three main regions of LDH activity, each region containing a relatively constant percentage of the total activity; in certain disease states such as leukemia and myocardial infarction the normal relationship became altered in a characteristic way (Vesell and Bearn, 1957). At that time there existed in the literature approximately 20 examples of enzyme heterogeneity, which had been usually regarded as artifactual, arising during purification procedures. Thus, in a discussion of clinical applications of the heterogeneity of lactic and malic dehydrogenase activities in human plasma and red cells (Vesell and Bearn, 1958), the following examples were gathered from the literature: pepsin (Desreux and Herriott, 1939), chymotrypsin (Kunitz, 1938), cytochrome C (Paléus and Neilands, 1950), ribonuclease (Hirs, Stein and Moore, 1951), lysozyme (Tallan and Stein, 1951), enolase (Malmström, 1957), xanthine dehydrogenase (Mitidieri, 1955) and LDH (Meister, 1950; Neilands, 1952). Now, only 7 years later, there are more than 100 examples of enzymes that have been demonstrated to exist in multiple molecular forms. Although a survey of these isozymes and of the biological contexts in which they were investigated provides material for a valuable review (Wieland and Pfleiderer, 1963), the purpose of this chapter is to present the genetic as well as some more general biological implications of recent studies on the most thoroughly investigated example of an enzyme manifesting multiple forms, the example of LDH. Because more information has been accumulated for LDH isozymes than for the multiple forms of any other enzyme, it is hoped that studies of LDH isozymes may serve as models for guiding and facilitating investigations of other systems.

Chemical Studies of LDH Isozymes

A. *Methodological Considerations*

Several forms of electrophoresis have been employed since 1950 to investigate LDH preparations from various mammalian sources. Moving boundary electrophoresis revealed two main peaks of beef heart LDH (Meister, 1950; Neilands, 1952). Starch block electrophoresis exhibited heterogeneity of human serum and erythrocyte LDH activity (Vesell and Bearn, 1957). Paper electrophoresis disclosed five bands of LDH activity in several tissues of the rat (Wieland and Pfleiderer, 1957). Agar gel electrophoresis offered certain advantages for rapid clinical determination of the LDH isozymes in human serum and tissues (Wieme, 1959). Starch gel electrophoresis was initially employed for investigating changes in LDH isozymes during development, and for comparative phylogenetic studies (Markert and Møller, 1959). Recently acrylamide gel electrophoresis has been introduced as a rapid and powerful technique suitable for demonstrating LDH isozymes (Ornstein, 1964). Cellulose acetate is also a satisfactory supporting medium for the demonstration of LDH isozymes in human serum (Preston et al., 1965).

Starch gel electrophoresis has proved so convenient and reproducible a method for the resolution of mixtures containing LDH isozymes and for screening large populations for genetic variants of LDH that this technique has been utilized more frequently than any of the others. The isozymes are readily visualized on the gel by incubating the gel slices for one-half to one hour in the dark at room temperature with a solution composed of the following: sodium lactate 60 per cent solution diluted four parts to six parts H_2O, 1.0 ml.; phenazine methosulfate 2 mg./ml., 0.4 ml.; β-nicotinamide adenine dinucleotide, 25 mg.; Nitro-blue tetrazolium, 19 mg.; sodium cyanide 0.06M, 2.0 ml.; tris buffer pH 7.5, 0.05M, 35.0 ml. Lactate is the substrate; nicotine adenine dinucleotide (NAD) is the coenzyme; the yellow colored nitro-blue tetrazolium becomes deposited as a deep purple-blue formazan at the site of lactate oxidation on the gel; phenazine methosulfate is the electron transporter; and cyanide maintains an anaerobic environment. This method has been adapted from several described in the literature (Dewey and Conklin, 1960; Vesell, 1961; van der Helm, 1961; Ressler and Joseph, 1962) and is a modification of the technique introduced in 1959 by Markert and Møller.

Starch gel electrophoresis is an extremely sensitive technique for the resolution of mixtures of proteins (Smithies, 1959). Several purely methodological considerations significantly affect the LDH isozyme patterns observed (Vesell and Brody, 1964). For example, on dilution LDH

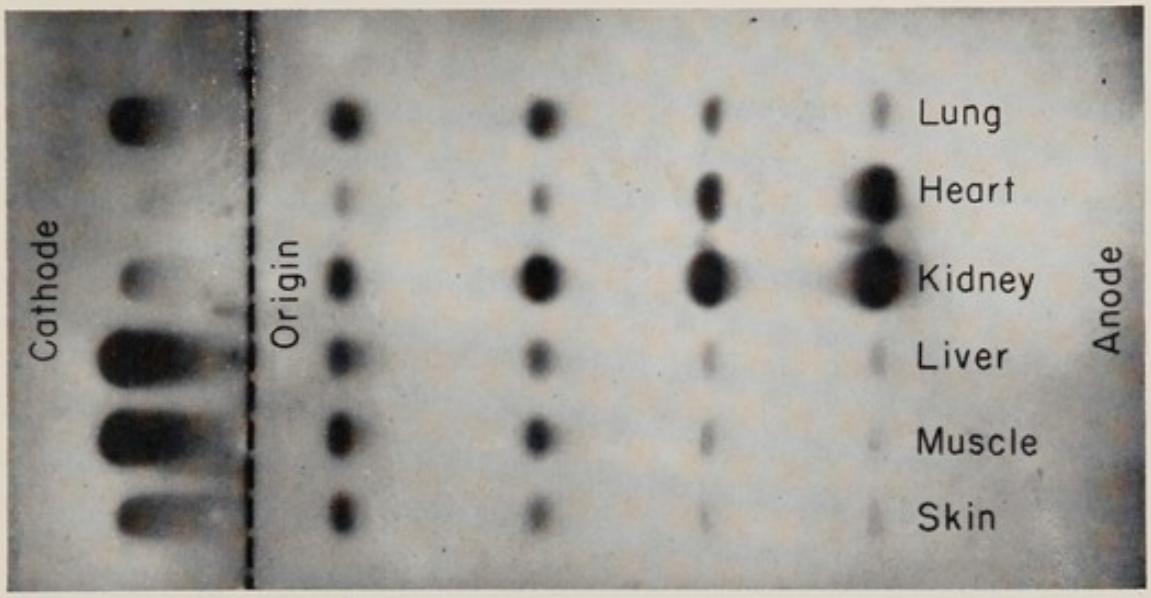

FIG. 1—Photograph of a starch gel treated to reveal LDH isozymes in homogenates of 6 human tissues obtained at autopsy (from Vesell et al., 1962a).

5 tends to disappear more rapidly on the gel than the other isozymes (Vesell, 1962; Ressler, Schulz and Joseph, 1963); and, furthermore, additional bands, the number and position of which vary, depending on the electrophoretic conditions, may frequently be seen to cluster about the main isozymes (Vesell and Bearn, 1962a, b; Blanco and Zinkham, 1963; Fritz and Jacobson, 1963; Costello and Kaplan, 1963; Markert, 1963a; Vesell and Brody, 1964).

Techniques other than electrophoresis have been utilized to isolate LDH isozymes. Hess and Walter (1960, 1961) employed DEAE cellulose column chromatography to separate five human LDH isozymes, as have Nisselbaum and Bodansky (1963). Reeves and Fimognari (1963) utilized DEAE cellulose column chromatography for isolation of pig heart LDH isozymes.

B. *LDH Isozyme Patterns in Normal Human Tissues*

On starch gel electrophoresis homogenates of human tissues yield isozyme patterns called zymograms shown in Figure 1. Figure 1 demonstrates several of the similarities and differences in isozyme patterns observed in human tissues (Vesell and Bearn, 1962a). In the six tissues illustrated in Figure 1 the zymograms resemble one another in containing five bands, four of which migrate toward the anode and one of which migrates toward the cathode. According to many physicochemical criteria, each of the LDH isozymes of one human tissue is identical to the isozyme of corresponding electrophoretic mobility in the other

tissues (Nisselbaum, Packer and Bodansky, 1964). What distinguishes one tissue from another is the distribution of total LDH activity among the five isozymes. Skin, muscle and liver exhibit most of their activity in the cathodal band (LDH 5), whereas heart and kidney contain most of their activity in the fastest migrating anodal bands (LDH 1 and LDH 2); lung is intermediate between these extremes. One genetic application involves Duchenne muscular dystrophy in which the carrier state was detected through reduction of muscle LDH 5 (Emery, 1964). The genetic problem posed by tissue specific isozyme patterns concerns the mechanism by which an organism, all of whose somatic cells have an identical genome, can develop and maintain such differences. Hypotheses designed to elucidate this enigma will be discussed in the section on genetic control (part A).

Before leaving the topic of the LDH isozyme pattern in normal human tissues, two exceptions to the observations described above should be mentioned. The first involves the isozyme pattern of mature erythrocytes and platelets. Both of these cells lack a nucleus and both also fail to exhibit LDH 5 by starch gel electrophoresis. Experimental evidence will be presented in the section on subcellular localization of LDH isozymes for a relationship between LDH 5 and the cell nucleus. Figure 2 demonstrates that the isozyme pattern of platelet preparations is also unusual in that a cathodal band fails to appear.

The second exception involves adult human testes and sperm. These tissues contain a sixth LDH isozyme migrating electrophoretically between LDH 3 and LDH 4 (Blanco and Zinkham, 1963; Goldberg, 1963). This sixth LDH isozyme, absent in prepubertal testes, has been termed the "X" band; it occurs in sperm from several different vertebrates, but its electrophoretic position varies with the species studied, some species exhibiting more than one "X" band, others revealing no distinctive testicular bands (Zinkham, Blanco and Kupchyk, 1963).

C. *Physicochemical Properties of LDH Isozymes*

The LDH isozymes have been shown to differ from one another in a variety of ways. These include affinity for substrates (Plagemann, Gregory and Wróblewski, 1960a; Vesell and Bearn, 1961; Nisselbaum, Packer and Bodansky, 1964), behavior toward coenzyme analogs (Vesell, 1961; Kaplan, 1964; Nisselbaum, Packer and Bodansky, 1964), sensitivity to inhibitors (Wieland and Pfleiderer, 1957; Plummer and Wilkinson, 1961; Emerson, Wilkinson and Withycombe, 1964; Brody, 1964), pH optima (Vesell and Bearn, 1958), immunological specificity (Nisselbaum and Bodansky, 1959, 1963; Plagemann, Gregory and Wróblewski, 1960b; Cahn et al., 1962; Markert and Appella, 1963) and thermal

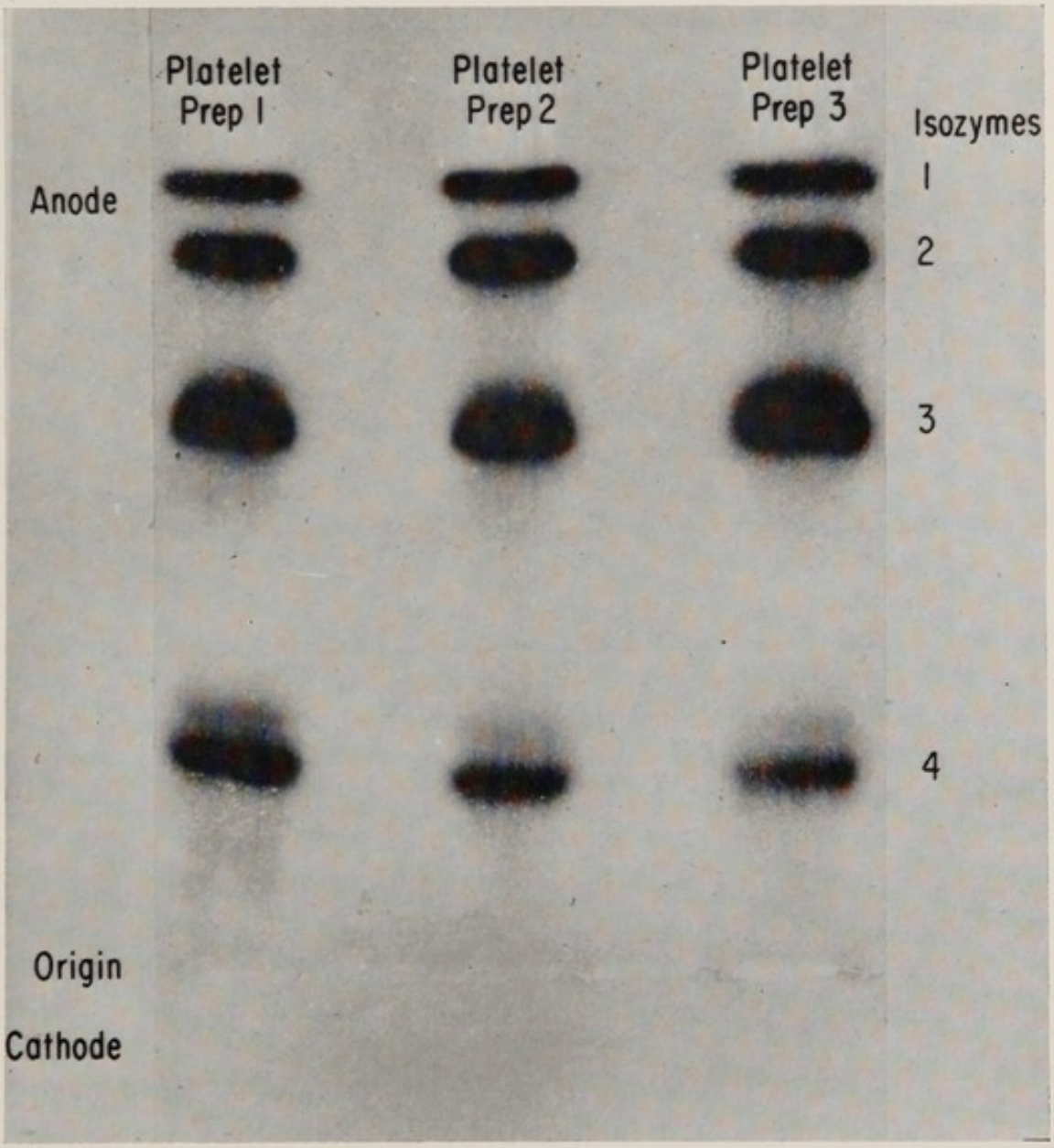

FIG. 2—Photograph of a starch gel illustrating the 4 LDH isozymes in human platelets. Note absence of LDH 5. The platelets obtained from three normal individuals were prepared by differential centrifugation. Prep 1 contained 222 leukocytes/mm^3 and 2 x 10^6 platelets/mm^3; prep 2 contained 4800 leukocytes/mm^3 and 2 x 10^6 platelets /mm^3; and prep 3 contained 1110 leukocytes/mm^3 and 2.8 x 10^6 platelets/mm^3.

stability (Plagemann, Gregory and Wróblewski, 1961; Zondag, 1963). Wieland and Pfleiderer (1961) demonstrated marked differences in amino acid composition of 4 LDH isozymes from rat heart; stepwise increments of aspartic acid residues occurred in order of increasing electrophoretic mobility toward the anode. These increments were accompanied by stepwise decrements in arginine and lysine residues.

But, according to many physicochemical parameters, the LDH iso-

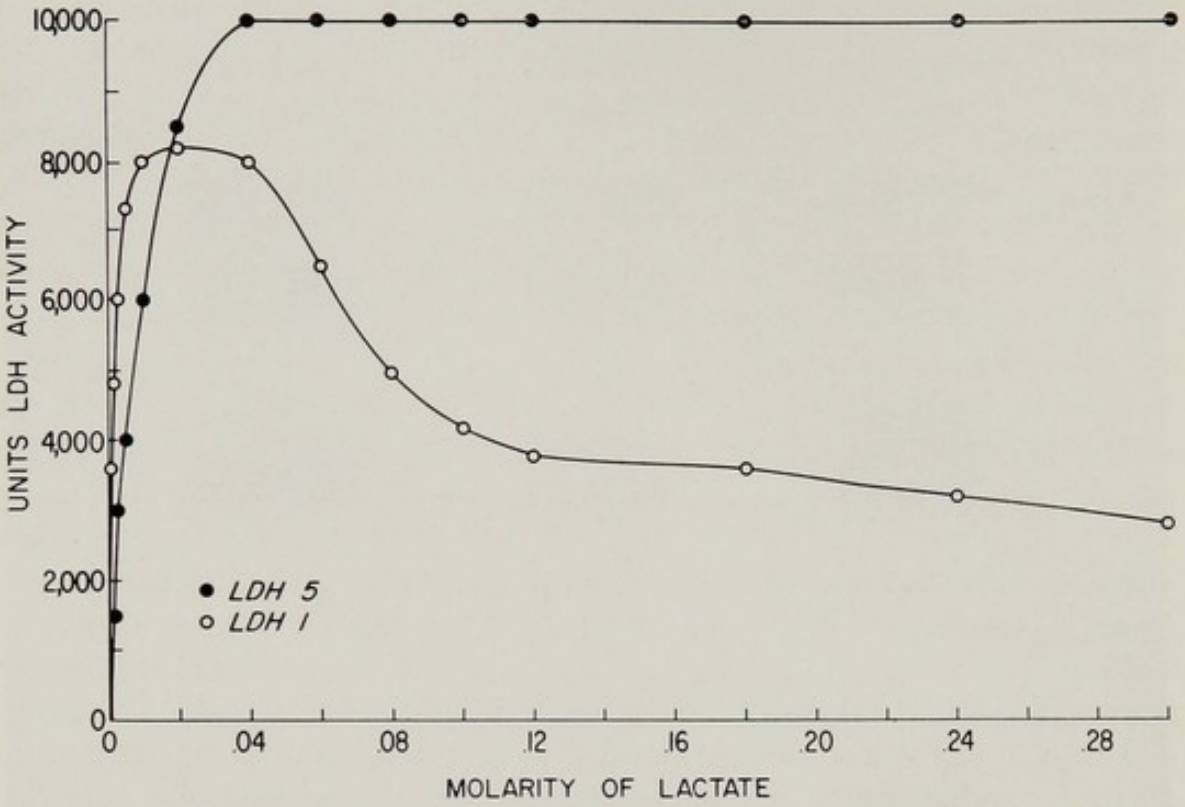

FIG. 3—Inhibition of LDH 1 by increasing concentrations of lactate which fail to inhibit LDH 5. K_MLACTATE for LDH 5 is 8 x 10^{-3}; K_MLACTATE for LDH 1 is 1 x 10^{-3}.

zymes behaved remarkably alike as exemplified by an investigation of the two main isozymes of beef heart (Markert and Appella, 1961). These two isozymes were indistinguishable in sedimentation coefficient, diffusion coefficient, partial specific volume, molecular weight, and frictional ratio.

One of the most interesting distinguishing features of the LDH isozymes is their differential inhibition by substrate. Even before separating their crude homogenates into isozymes, Kaplan and Ciotti (1961) suspected LDH heterogeneity because the extracts of one tissue of an organism varied in affinity for substrate and coenzyme from extracts of other tissues of the same organism. Pfleiderer and Wachsmuth (1961) called attention to the resistance of LDH 5 to the inhibitory effects of lactate on LDH 1. They, therefore, suggested that LDH 5 predominated in such anaerobic tissues as liver and skeletal muscle because it could function efficiently in the presence of high lactate concentrations and that LDH 1 predominated in such aerobic tissues as brain and heart where lactate accumulation was minimal. Figure 3 illustrates the resistance of human LDH 5 to lactate concentrations which inhibit LDH 1. Figure 4 shows the inhibitory effect of high concentrations of pyruvate

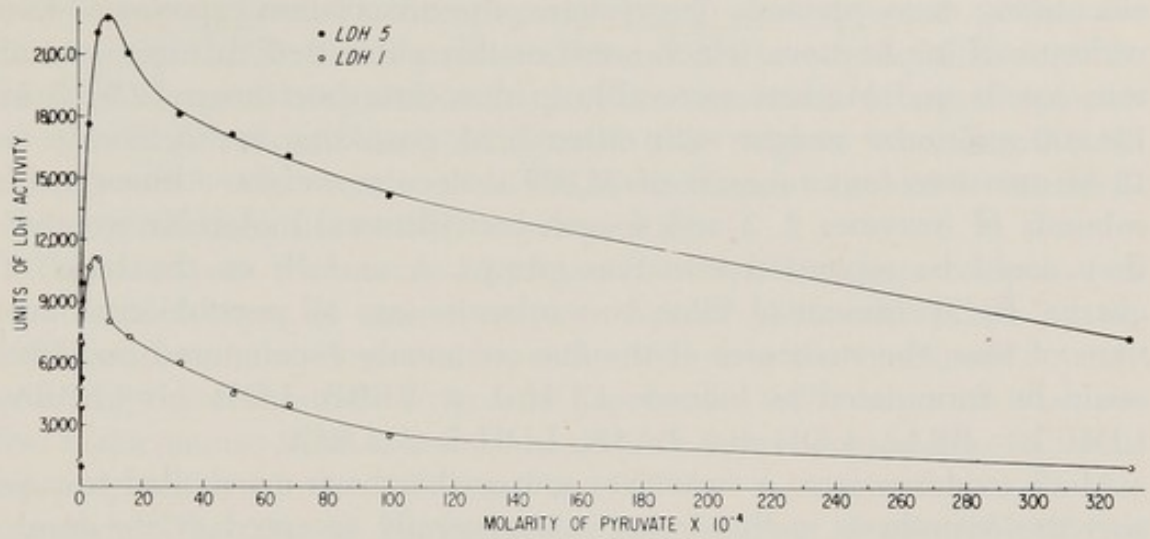

FIG. 4—Inhibition of both LDH 5 and LDH 1 by increasing concentrations of pyruvate. K_MPYRUVATE for LDH 5 is 9.6 x 10^{-5}, for LDH 1 is 5 x 10^{-5}.

on both LDH 5 and LDH 1, LDH 1 being inhibited to a greater extent than LDH 5 by a given concentration of pyruvate. The hypothesis of LDH 5 predominance in tissues most dependent on anaerobic metabolism has been invoked in an interpretation of the observations that different human skeletal muscles vary in isozyme pattern (Kar and Pearson, 1963) and also that identical muscles from different species exhibit different isozyme patterns (Kaplan, 1964). These species differences in isozyme pattern have been correlated with evolutionary alterations in the physiological role subserved by a specific muscle.

One of the main functions of LDH is to regulate the intracellular ratio of NAD to $NADH_2$. This ratio is maintained above 1, both by the establishment of an enzyme-independent equilibrium constant for the reaction which is 10 times more favorable for NAD than for $NADH_2$ formation, as well as by the greater affinity of LDH for pyruvate (approximate K_M 10^{-5}) than for lactate (approximate K_M 10^{-3}). The 5 LDH isozymes vary in affinity for each of these two substrates; therefore, by virtue of its specific location intracellularly, each isozyme may serve to develop slightly different ratios of NAD to $NADH_2$ within various areas of the cell.

D. *Subunit Structure of LDH Isozymes*

One of the most far reaching discoveries in the past few years in the field of LDH isozymes was the formulation of their structure based on the dissociation of a beef heart isozyme (LDH 1) into 4 subunits (Appella and Markert, 1961). In addition to providing a new hypothesis

elucidating many previous observations, the dissociation experiment had widespread implications which were readily submitted to experimental test. Apella and Markert were able to dissociate beef heart LDH 1 of 134,000 molecular weight with either 5 M guanidine hydrochloride or 12 M urea into four subunits of 34,000 molecular weight. Although the subunits of isozymes 2, 3 and 4 each had identical molecular weights, they could be separated into two groups, A and B, on the basis of charge. By assortment of these two subunits into all possible combinations of four, the structures of the five commonly encountered isozymes could be formulated as follows: LDH 1 = BBBB, LDH 2 = BBBA, LDH 3 = BBAA, LDH 4 = BAAA, LDH 5 = AAAA.

Much evidence from a variety of sources has been marshalled to support this hypothesis so that today it is generally accepted (Cahn et al., 1962). Thus far it appears that many dehydrogenases known to exist in multiple forms differ in structural basis from the LDH system; alcohol dehydrogenase (Kägi and Vallee, 1960), glyceraldehyde-3-phosphate dehydrogenase (Harris and Perham, 1963), glutamic dehydrogenase (Yielding and Tomkins, 1960), as well as enolase (Deal et al., 1963; Stellwagen and Schachman, 1962) and aldolase (Westhead, Butler and Boyer, 1963) are polymers, each enzyme being an aggregate of only one type of subunit.

Evidence in support of the subunit hypothesis for LDH structure includes the observation that the number of peptides resulting from complete trypsin digestion is approximately one-fourth the number of arginine plus lysine residues found on total amino acid analysis (Appella and Markert, 1961; Markert, 1963b). Also, immunological evidence shows that antibody made to LDH 1 will not crossreact with LDH 5 but will crossreact in diminishing amounts with LDH 2, 3 and 4, in quantities predicted from the proposed structures for the five isozymes. Similarly, antibody to LDH 5 will not react with LDH 1 but will react with LDH 2, 3 and 4 to the extent expected (Cahn et al., 1962; Nisselbaum and Bodansky, 1963; Kaplan and White, 1963; Nace, 1963; Markert and Appella, 1963).

Several different genetic studies on mutations affecting the synthesis of LDH subunits provide additional support of the hypothesis and will be discussed in the section on genetic control (part B).

Perhaps the most compelling evidence in favor of the subunit hypothesis involves recombination of the subunits to form all five isozymes (Markert, 1963a). Markert isolated isozymes 1 and 5 from beef tissues by chromatography and electrophoresis; he then mixed them in equal proportions in 1 M NaCl, froze them overnight and after the mixtures

were thawed, resolved them by starch gel electrophoresis. Since the structure of LDH 1 was predicted to be A^0B^4 and of LDH 5, A^4B^0, Markert expected dissociation to produce a mixture of equal amounts of A and B subunits and anticipated that random recombination of the subunits would occur to yield the five isozymes in a ratio of 1:4:6:4:1. These predictions he verified experimentally.

The discovery that many LDH isozymes are tetramers composed of two distinct types of monomers was rapidly confirmed (Cahn et al., 1962) and stimulated several investigations into the biochemical properties of these subunits. Fritz and Jacobson (1963) reported that the five major mouse LDH isozymes could be subfractionated into 15 bands by preparation of the starch gel in .005M β-mercaptoethanol. It was postulated that the 15 bands result from binding of one molecule of NAD to each of the monomeric subunits and that mercaptoethanol removes NAD selectively from the A subunit. Therefore, the five bands appearing in the LDH 5 region corresponded to forms whose structures might be indicated as follows: A^4N^4, A^4N^3, A^4N^2, A^4N^1, A^4, where N represents the NAD molecule. Costello and Kaplan (1963) also described 15 bands of LDH activity in mouse tissues but hypothesized the existence of two distinct types of A subunits, which they designated M^A and M^B. On random recombination these two subunits would yield the five bands seen in the LDH 5 position ($M^AM^AM^AM^A$, $M^AM^AM^AM^B$, $M^AM^AM^BM^B$, $M^AM^BM^BM^B$ and $M^BM^BM^BM^B$). Thus far no definitive evidence to support either the theories of Fritz and Jacobson or of Costello and Kaplan has been presented.

Initially the conditions for renaturation of the monomeric subunits of LDH produced by treatment with 5 M guanidine hydrochloride or 12 M urea were not found (Appella and Markert, 1961; Markert, 1963a). However, Epstein and co-workers (1964) demonstrated that the higher orders of structure of the LDH molecule were determined by the primary structure of the subunits, since reconstitution of the isozymes of rabbit muscle LDH was clearly shown following disaggregation in urea.

Genetic Control of LDH Isozymes

A. *Mechanisms for the Genetic Control of Tissue Isozyme Patterns*

On the basis of the kinds of experiments performed by Appella and Markert (1961) and by Cahn et al. (1962) the existence of subunits A and B became firmly established. According to the current dogma requiring one genetic locus for each polypeptide chain, synthesis of these

two subunits was thought to be directed by two separate genetic loci designated a and b. Extension of this concept provided an explanation for tissue specific isozyme patterns. Thus in tissues where the activity of gene a was greater than the activity of gene b, subunit A would have preponderance over subunit B, resulting in production of more isozyme 5 (A^4B^0) and 4 (A^3B^1) than of 1 (A^0B^4) and 2 (A^1B^3). Conversely, isozymes 1 and 2 would be most abundant in tissues where the activity of gene b exceeded that of gene a. Although Markert stated (1963a) that by shifting the proportions of isozymes 1 and 5 in the mixture before freezing, the resulting patterns could be skewed to generate the various isozyme distributions observed in normal tissue homogenates and although Markert (1963b) published predictions of what isozyme patterns would result from such mixtures, no demonstration has thus far appeared to show whether isozyme patterns produced *in vitro* resemble those observed *in vivo*.

Figure 5 reveals that the extreme patterns of human liver and muscle on the one hand, and of erythrocytes and kidney on the other, can in fact be formed *in vitro* by mixing appropriate proportions of LDH 1 and LDH 5. This experiment lends support to the contention that the isozyme patterns of different tissues may arise *in vivo* from random recombination of subunits and that the number of available A subunits relative to the number of available B subunits governs the final pattern. Isozymes 1 and 5 were isolated by starch block electrophoresis of homogenates of human heart and psoas muscle obtained at autopsy. Electrophoresis was performed in phosphate buffer pH 7.4, 0.05M; and the sections of the block on which 15 ml. of heart and 15 ml. of muscle homogenate had been separated were eluted with either 1.5 ml. of 1 M NaCl or 1.5 ml. of 1 M sodium phosphate. LDH 1 and LDH 5 were mixed in activity ratios of 3:3, 1:10 and 10:1 and the mixtures frozen for 4 hours at −20°C. Figure 5 reveals that a mixture of 10 parts LDH 1 activity to 1 part LDH 5 activity will produce an isozyme pattern resembling that of human kidney. This 10:1 ratio must be increased to approximately 30:1 to achieve the isozyme pattern of heart. Figure 5 should be compared with Figure 1. A pH in the physiological range of 7.4 was critical for recombination, since, following isolation of LDH 1 and LDH 5 on a barbital block pH 8.6, recombinants 2, 3 and 4 could not be obtained after freezing. However, dissociation and recombination were achieved from material obtained on a starch block prepared in barbital buffer pH 7.5. This observation, together with failure to achieve recombination at pH less than 6.0, suggests that the environmental requirements for random recombination of the subunits *in vitro*

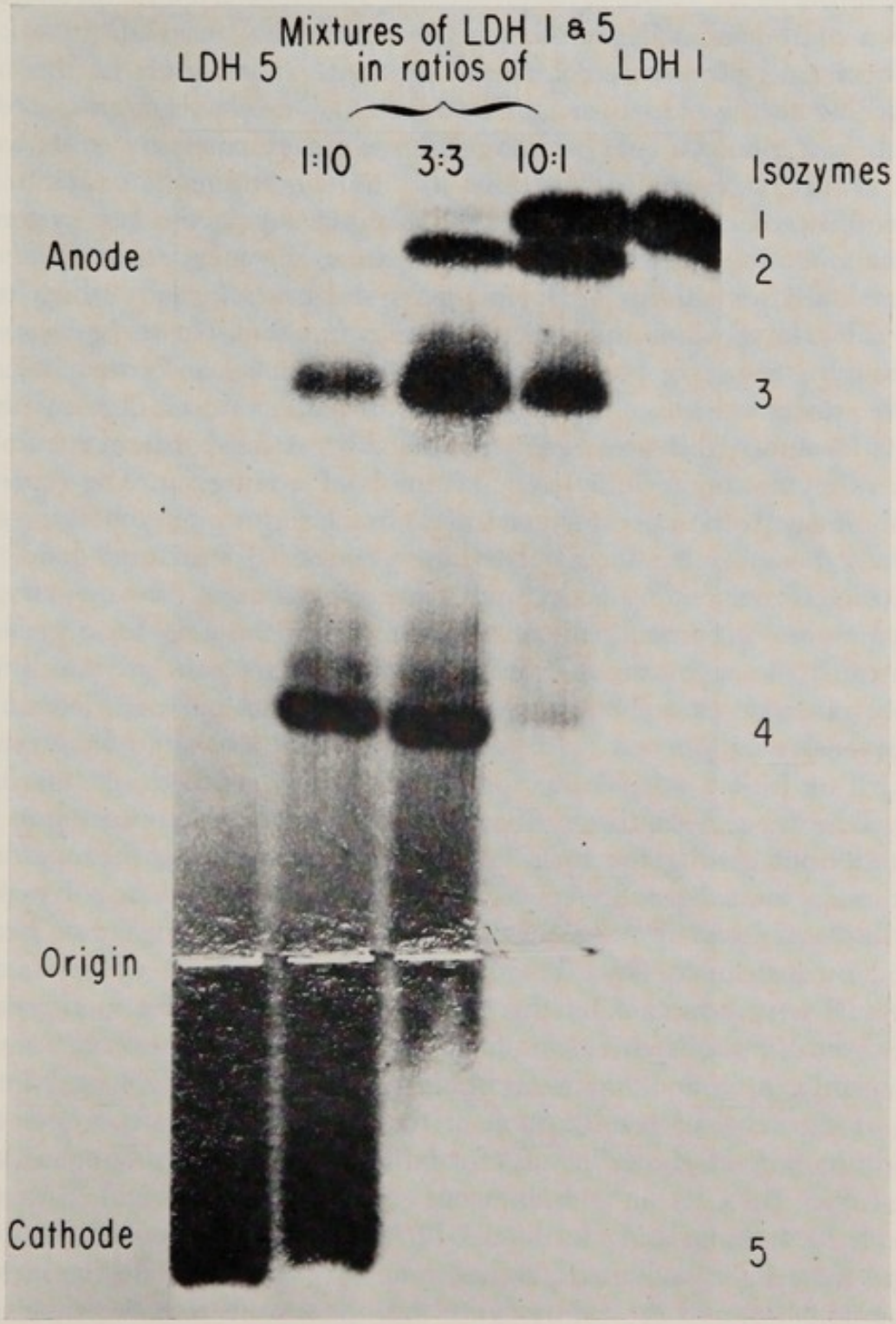

FIG. 5—Photograph of a starch gel illustrating the isozyme patterns obtained following recombinations of mixtures containing varying proportions of LDH 1 and LDH 5. The human isozymes 5 and 1 were obtained by elution with 1M NaCl from a starch block prepared in barbital buffer pH 7.5, 0.05 M. Mixtures of LDH 5 and LDH 1 in ratios of 10:1, 3:3 and 1:10 were frozen for 4 hrs., thawed and the recombinant patterns are shown here (from Vesell, 1965a).

are narrow as far as pH is concerned but are satisfied by the pH existing within most mammalian cells.

The results of the recombination experiments will now be discussed according to the model for genetic regulatory mechanisms proposed by Jacob and Monod (1961), although no evidence currently exists to relate them and such a discussion is entirely speculative. Jacob and Monod, based on their intensive study of the β-galactosidase system in *Escherichia coli*, distinguished two varieties of genes: *structural genes* which initiate synthesis of polypeptides and *control genes* which regulate the rate at which the structural genes function. *Control genes* were subdivided into two types: *operator genes* which are located adjacent to the structural genes and directly control the rate of their synthetic activities and *regulator genes* which, located at some distance from the genes they act upon, initiate the synthesis of a repressor. The repressor combines with the operator gene, thereby closing it. The operator gene is limited in that it affects only the adjacent *(cis)* structural genes and not the corresponding structural genes located on the homologous chromosome in diploid organisms. However, the regulator gene by means of the repressor acting through the cytoplasm of a cell may either open or close both *cis* and *trans* operator genes. Whether the operator gene is opened or closed depends on the nature of the repressor with which a specific cytoplasmic metabolite called *effector* interacts. One type of repressor through inactivation by its effector is prevented from closing the operator gene, thus permitting the operon to synthesize messenger RNA with eventual formation of a polypeptide and *induction* of enzyme synthesis. The other type of repressor is activated by its effector and directly blocks and closes its operator; messenger RNA production by this operon is thus stopped and *repression* of enzyme synthesis occurs.

Recently, attention has been refocused from repression and induction at the level of transcription of messenger RNA on the operon to their function at the level of translation where a single molecule of messenger RNA is in simultaneous contact with several ribosomes (Stent, 1964; Ames and Martin, 1964). This moment of contact between single messenger and multiple ribosomes provides with its implication of motion between the participants the opportunity for the translation process to end before completion of the reading. Ames and Martin (1964) suggested this mechanism to explain operon *polarity* in which a single mutant results in diminution of activities of several enzymes distal to it on an operon but none proximal to it. The model has also been invoked to elucidate *modulation*, which is genetic maintenance of dif-

ferential rates of polypeptide synthesis through coding for transfer RNA species of different abundance. That is, should the right aminoacyl-transfer RNA be available for incorporation into the polypeptide being synthesized on a ribosome, the translation process will most likely proceed to the next residue; should it not be available, the probability of continuation is low (Stent, 1964; Ames and Martin, 1964). The theory of modulation predicts that fewer polypeptides are made with movement down the operon and that an evolutionary selection occurs in the order of genes on the operon from least efficient of which more are required to most efficient which are furthest away from the operator. The virtue of this model is that it can be effectively applied to many biological systems (Neel, 1961; Ceppellini, 1961; Motulsky, 1962; Fudenberg and Franklin, 1963; Parker and Bearn, 1963; Watson et al., 1964).

Tissue differences in isozyme patterns may also be interpreted in the light of these ideas. It may be postulated either that operator genes in different cells of an organism exert their control over the LDH structural genes at different rates or that the translation process for producing polypeptides A and B proceeds at different rates on the ribosomes of various cells. Since the rate of activity of the operator gene is determined by the interaction of repressor with specific cytoplasmic factors, it may be postulated that these cytoplasmic factors would vary from one tissue of an organism to another. The model is very flexible and can also accommodate the unlikely possibility that the a and b structural genes may be located in different operons and thus under the control of 2 different operator genes. This separation would provide an explanation for the fact that the a and b structural genes in mammalian tissues rarely seem to produce A and B subunits at equal rates. If equal amounts of A and B subunits were available intracellularly for random recombination, then the resulting isozymes from 1 to 5 would be expected to display a ratio of 1:4:6:4:1, whereas most human tissues exhibit preponderance of either isozyme 1 or isozyme 5, but rarely of isozyme 3.

A mechanism explaining the evolution of two distinct operons for the a and b genetic loci would be chromosomal duplication, in which during crossing-over a fragment breaks off and is repeated later in the chromosome. A mutation might affect one of the duplicated pieces at a structural gene locus to induce the change from structural gene a to structural gene b. This mechanism will be reconsidered in the section on phylogenetic studies. A regulator or operator gene mutation would affect the total quantity of LDH available intracellularly and has as yet

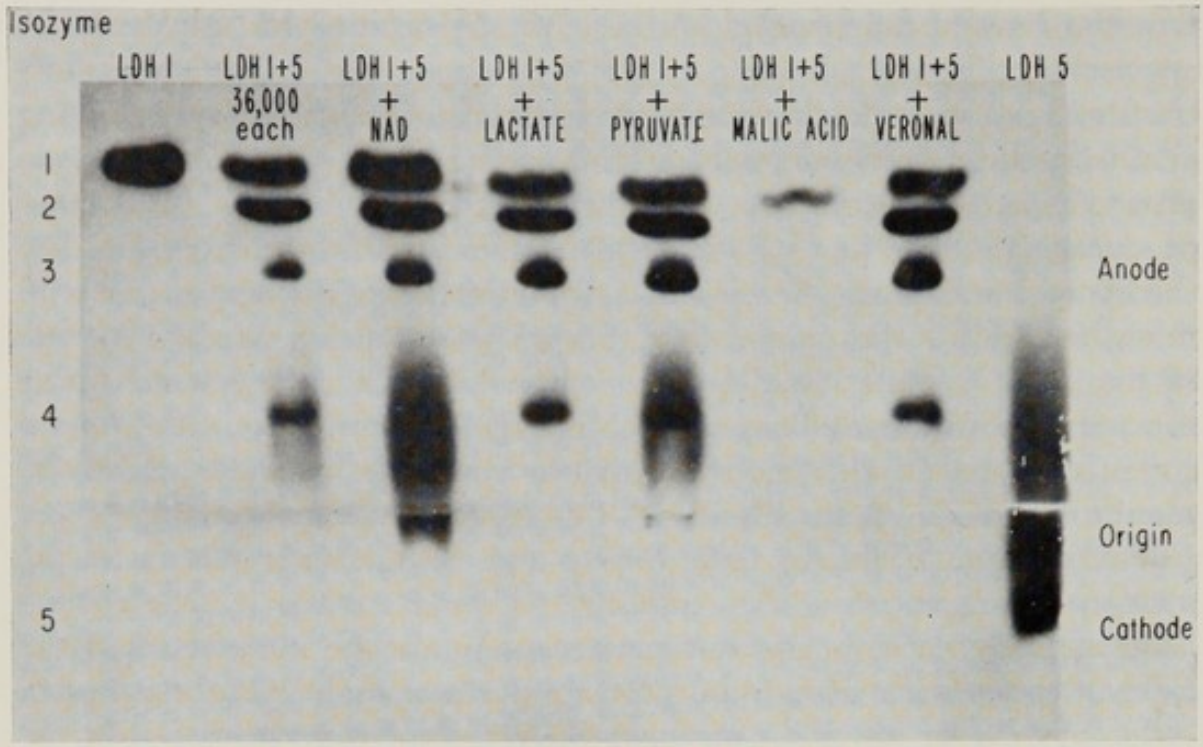

FIG. 6—Photograph of a starch gel illustrating isozyme patterns resulting from recombination of LDH 1 and LDH 5 in 1:1 ratio of activity. This pattern is compared to those obtained when final concentrations of 10^{-3} M NAD, 10^{-3} M lactate, 10^{-3} M pyruvate, 10^{-3} M malic acid and 0.05 M veronal buffer were each separately added to the mixture. Note that these reagents in high concentrations failed to alter significantly the recombinant isozyme pattern except in the case of malic acid where the pH of the mixture prior to freezing was 6.4 instead of 7.4, as in the others. When the pH of the malic acid solution was raised to 7.4, the recombinant isozyme pattern resembled that of the others.

not been described, but a structural gene mutation would affect the type of subunit synthesized and such mutations have been reported and will be discussed at length in the section on genetic control (part B). Finally, differential intracellular catabolism of LDH 1 and LDH 5 may play a role in their relative abundance in various tissues, and on this aspect of isozyme control no information is currently available.

Now that differential gene activity influenced to some degree by the cytoplasmic environment has been discussed as a possible mechanism for determining the tissue isozyme patterns, the extent to which specific epigenetic factors modify the isozyme pattern will be considered. In an attempt to define some cytoplasmic conditions which might influence the isozyme pattern obtained on recombination of the A and B subunits certain substrates and coenzymes were added to mixtures of LDH 1 and LDH 5 just prior to freezing and alterations in the resulting isozyme patterns were sought. A variety of compounds were added to mixtures containing equal activities of LDH 1 and LDH 5 to yield the following

final concentrations: 10^{-3}M lactate, 10^{-3}M pyruvate, 10^{-3}M NAD, 10^{-3}M NADH, 10^{-3}M malate, .05M veronal buffers at pH 7.4 and at pH 8.6. The mixtures were frozen for 4 hours, thawed and then compared on starch gel electrophoresis to a similarly treated mixture containing only equal activities of LDH 1 and LDH 5. The results are shown in Figure 6. All patterns are similar except for the malate experiment in which the pH of the mixture was not 7.4, as in the others, but 6.4. This result is in harmony with the veronal experiments in that dissociation of LDH 1 and LDH 5 into subunits and recombination of the subunits occurred if veronal buffer pH 7.4 was employed, but not when veronal buffer pH 8.6 was used. Therefore, the pH range required for recombination appears to be quite narrow.

Recent intensive studies of disaggregation or at least hybridization of LDH isozymes on freezing have indicated the complexity of the critical conditions including such factors as rapidity of freezing and thawing, increased salt and protein concentration during freezing, decreasing pH near the eutectic point of the salt, specific ion effects, weakening of hydrophobic bonds in the isozymes and freezing out of bound water at low temperatures (Chilson et al., 1965a, b). Thus special effects observed with the freezing technique, such as protection against denaturation by polyhydroxy compounds, may not be obtained under other circumstances (Vesell, 1965a). Similarly, the technique described by Epstein et al. (1965) exposes isozymes to urea and to extensive dilution and reconcentration, conditions which may differentially affect certain isozymes (Brody, 1964; Vesell, 1962).

Broad interpretation of these results suggests that transient variations in the concentration of certain common metabolites do not induce changes in the cellular isozyme pattern. However, certain more drastic alterations in the cellular environment will change the isozyme pattern, and these conditions will be discussed in the section on environmental conditions. Furthermore, certain common metabolites, as yet untested, may prove influential in altering the isozyme pattern produced on recombination of A and B subunits. It should be mentioned that the kinetics of recombination of A and B subunits have not been studied, and the current experimental techniques of recombination involving several hours of freezing or of extensive dilution and reconcentration following denaturation with 10.5 M urea or 5 M guanidine hydrochloride (Epstein et al., 1964) handicap kinetic analysis. Once this difficulty is overcome and relative rates of formation of the five isozymes from component subunits are ascertained, the effect of various reagents in accelerating or retarding isozyme production may be investigated. In this connection the allosteric model proposed by Monod and co-workers

(1962) might be mentioned. Allosteric sites are positions on a protein other than the active site. The small molecules attached to allosteric sites differ in character from the substrate or substrate analogues. The kinetic properties of the active site of a protein are, however, markedly altered by attachment of molecules at the allosteric site. The LDH molecule has as yet not been investigated for possible allosteric sites, and it may be that sufficient biological variability had been achieved for the pyruvate to lactate reaction by the availability of multiple molecular forms of LDH. On the other hand, the structure and function of the LDH molecule may prove to be as subject to allosteric control as the structure and function of another dehydrogenase, glutamic dehydrogenase, which becomes disaggregated into subunits on treatment with several steroid hormones (Yielding and Tomkins, 1960).

In addition to differential rates of activity of structural genes a and b and to the influence of certain epigenetic factors the possibility that the control mechanism for the tissue isozyme pattern depends upon the precise quantity of subunits available for recombination should be considered. This hypothesis predicts that different isozyme patterns would result from varying the total level of subunits present intracellularly even though the ratio of A to B subunits is held constant. An experiment was therefore performed in which isozymes 1 and 5, fixed in a 1-to-1 ratio, were mixed in serial dilutions. The results shown in Figure 7 demonstrate that below a certain relatively high activity of LDH 1 and LDH 5 in the mixture recombination will not occur. Furthermore, they reveal that where recombination does occur the resulting isozyme patterns vary only very slightly with different total LDH activities present in the mixtures. The proposed control mechanism tested in these experiments would permit the production of different tissue isozyme patterns according to the quantity of equimolar amounts of A and B subunits available in the tissue. Consistent with this possibility is the fact that there are marked differences among human tissues in total LDH activity (Wróblewski, 1958). Such a mechanism would obviate the necessity of differential gene activities in different tissues and permit the activities of structural genes a and b to remain fixed in relationship to each other. Against this third hypothesis is the fact that the *in vitro* model failed to provide sufficient diversity of isozyme patterns on recombination to approach the wide variety observed in human tissues (Fig. 7).

B. *Genetically Controlled Variants of LDH Isozyme Patterns*

Recent reports of mutations at the a or b genetic loci of the LDH subunits were largely directed toward gaining additional insight into

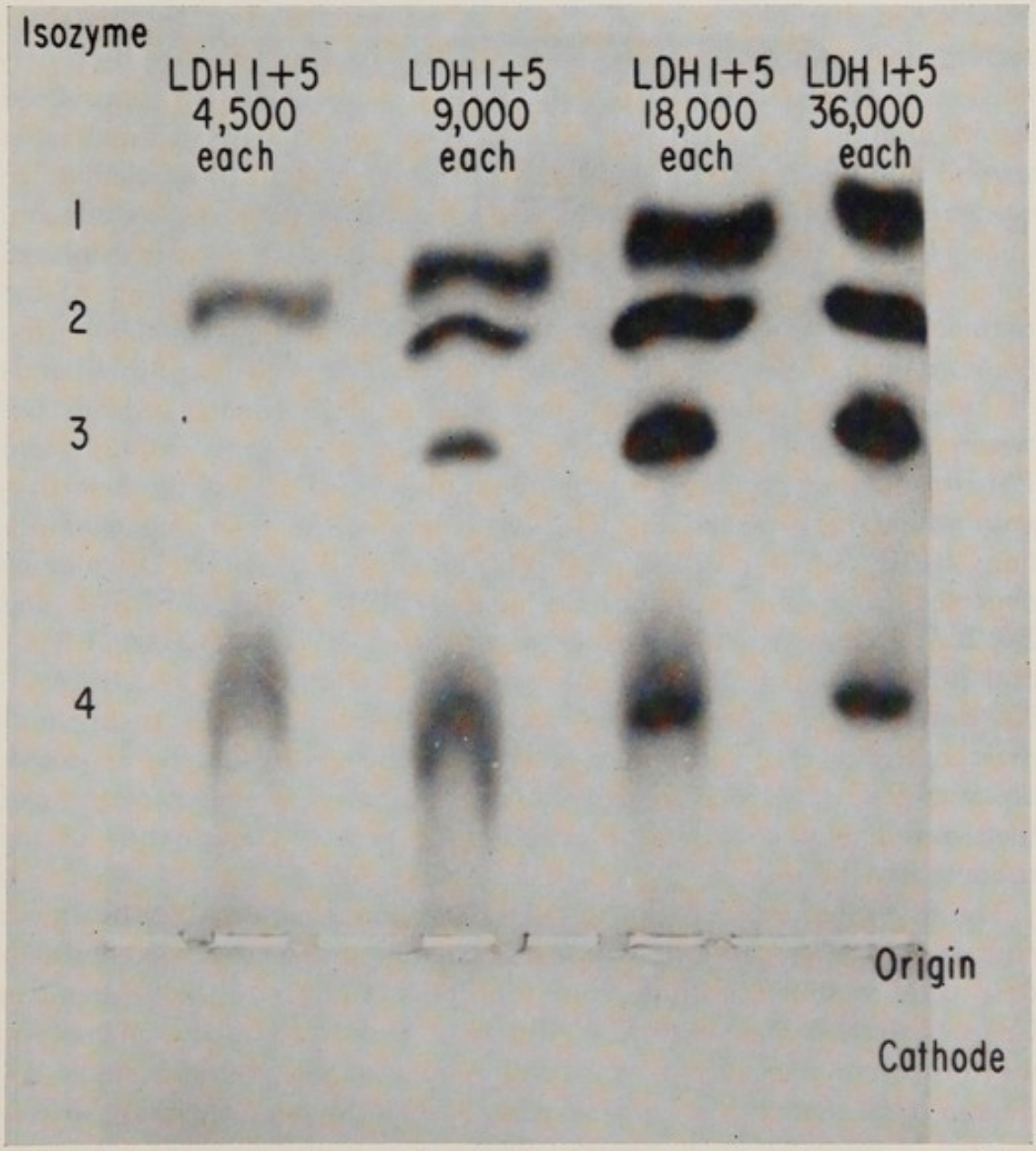

FIG. 7—Photograph of a starch gel illustrating recombination patterns of 1:1 mixtures of LDH 5 and LDH 1. These mixtures were diluted serially prior to freezing and the results show that below a certain concentration of LDH 1 and LDH 5 recombination is not obtained. At certain concentrations of LDH 1 and LDH 5 activity, more recombination—as judged by the intensity of LDH 3 compared to that of LDH 1—is achieved than at other activities. These differences in extent of recombination appear slight, but their tendency indicates decreased recombination with increasing dilution.

the tetrametric nature of isozyme structure (Shaw and Barto, 1963; Boyer, Fainer and Watson-Williams, 1963; Nance, Claflin and Smithies, 1963; Kraus and Neely, 1964). The deer mouse, *Peromyscus maniculatus*, was selected for screening studies. LDH isozyme patterns of liver, kidney, brain and testes from each animal were examined on starch

gels. When a mutant type was found in a related sibship, brother-sister matings of the offspring of the probands and backcrosses with both parents were carried out. The mutant allele was shown to be transmitted as an autosomal codominant. The heterozygous mouse demonstrated several additional bands of LDH isozymes 1, 2 and 3, intermediate in electrophoretic mobility between the normal and homozygous affected mice (Fig. 8). Both normal and homozygous affected animals exhibited single bands on the gel for isozymes 1, 2, 3 and 4; the position of the normal isozyme was ahead of the corresponding isozyme from the homozygous affected mouse. Heterozygous mice revealed multiple bands of intermediate mobility which were interpreted as being hybrids between the normal and mutant subunits. If B is the normal subunit, and B′ is the subunit produced by the mutant allele, LDH 1 of the heterozygote would be expected to yield five bands of the following composition: BBBB, BBBB′, BBB′B′, BB′B′B′, and B′B′B′B′; LDH 2 would be expected to yield four bands designated BBBA, BBB′A, BB′B′A and B′B′B′A; LDH 3 would be expected to reveal 3 bands (BBAA, BB′AA and B′B′AA) and LDH 2 would have two bands (BAAA and B′AAA). No differences in the electrophoretic mobility of LDH 5 among normals, heterozygotes and homozygous affected mice would be expected. Shaw and Barto (1963) demonstrated experimentally the correctness of these predictions and thus provided strong genetic confirmation of the subunit hypothesis.

The first mutation of a human LDH isozyme was described by Boyer and co-workers (1963). Erythrocytes from approximately 650 individuals were examined, a group comprised of 50 Caucasians, 300 American Negroes, 100 Papuans and 200 Nigerians. Only one mutant phenotype was observed; it occurred in a healthy 25 year old Nigerian male. Although a frequency for isozyme variants of 0.15 per cent may be calculated in this very heterogeneous group, it is probably more correct to indicate the frequency for each population so that for the 200 Nigerians the frequency was 0.5 per cent and for each of the other three groups it was 0 per cent. The mutation occurred in the B subunit and, as in the case of Shaw and Barto's mice, the heterozygote human had five bands in the LDH 1 position, four in the LDH 2 position, three for LDH 3, two for LDH 4 and one for LDH 5. The formulae for the isozymes, four of which are composed of hybrid combinations of mutant and normal subunits, proposed by Boyer et al. are identical to those suggested by Shaw and Barto. The total erythrocyte LDH activity of the Nigerian with the variant B subunit was comparable to that observed in normal erythrocytes, as were each isozyme's Michaelis-Menten constants and kinetic behavior with NAD analogues (Boyer, Fainer and Watson-

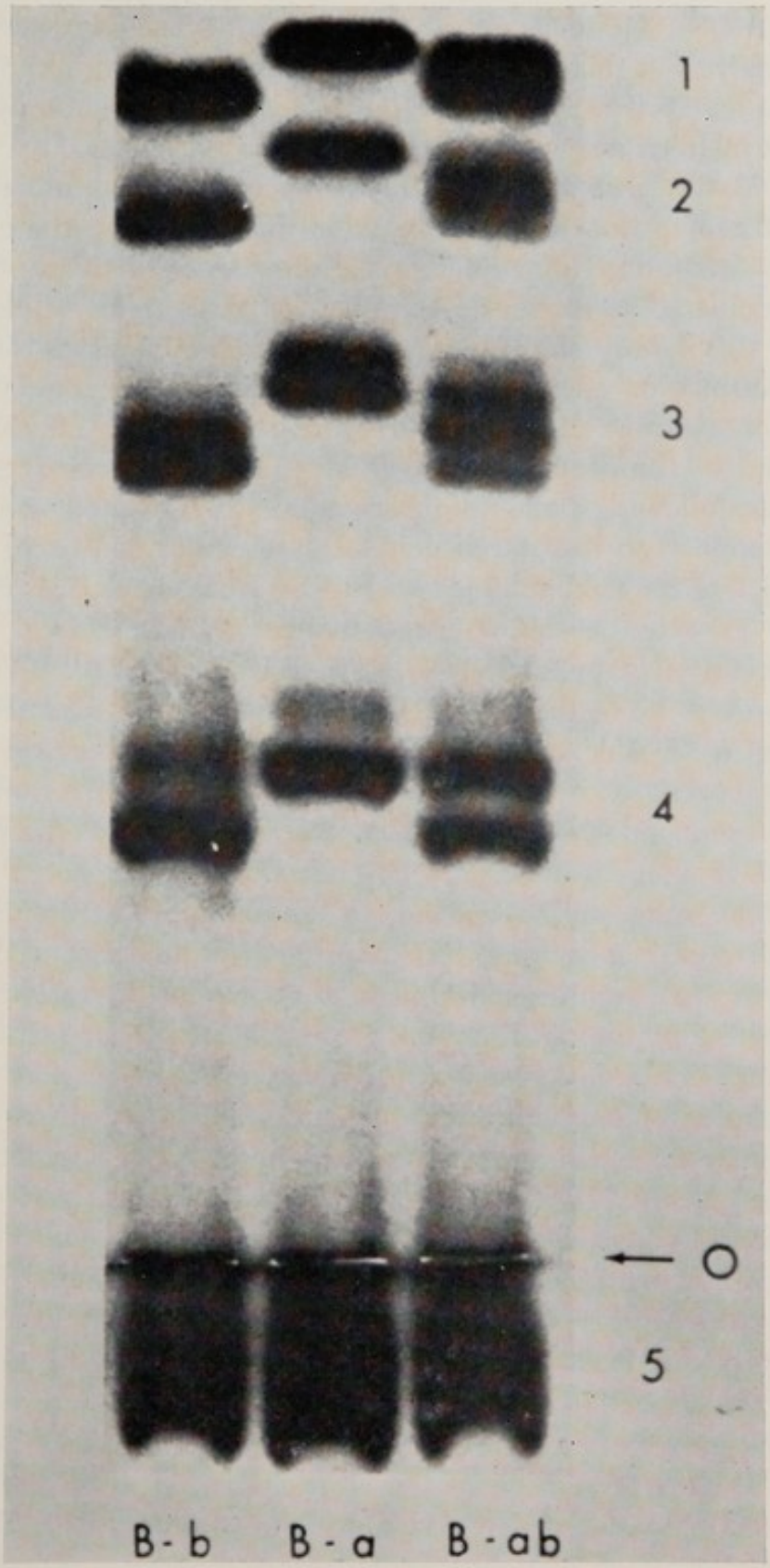

FIG. 8—Photograph of a starch gel illustrating three phenotypes of LDH isozymes in brain extracts from deer mice *Peromyscus maniculatus:* B-a normal; B-b homozygous variant; and B-ab heterozygous. Anode is at top, origin is at 0 (from Shaw and Barto, 1962).

Williams, 1963). No cells other than erythrocytes were subjected to isozyme analysis in this or the two subsequent papers describing human LDH variants. However, plasma from the affected individuals exhibited an isozyme pattern resembling that of their erythrocytes.

Nance, Claflin and Smithies, (1963) encountered a mutant a allele in the erythrocyte isozymes of a Brazilian family during the course of a genetic investigation. Unfortunately the prevalence of the mutation cannot be calculated because the number of individuals studied is not stated. In this family the mutation appeared in two generations, suggesting transmission as a codominant, and altered isozymes were identified in three of six siblings. Four individuals in the family were affected including the propositus. Given an individual heterozygous at the a allele, the following bands with structures as indicated would be expected to appear on the starch gel: LDH 1 would consist of one band (BBBB), LDH 2 would exhibit two bands (BBBA and BBBA′), LDH 3 would be expected to show three bands (BBAA, BBAA′, BBA′A′), LDH 4 should yield four bands (BAAA, BAAA′, BAA′A′, BA′A′A′) and LDH 5 would be expected to display five bands (AAAA, AAAA′, AAA′A′, AA′A′A′, A′A′A′A′). LDH 5 has not been observed on starch gel electrophoresis of hemolysates prepared from mature human erythrocytes (Vesell and Bearn, 1962a,b). Nance and co-workers failed to observe LDH 4 and found only two of the three expected bands for LDH 3. They suggested, according to reasoning based on distances in the gel between bands, that the hybrid corresponding to BBAA′ did not form in their family and hence that random association of the products of the normal and mutant alleles did not occur in this particular situation. Kraus and Neely (1964), however, in each of three different examples of mutants of the a gene which they discovered were able to identify three distinguishable bands in the LDH 3 region.

Kraus and Neely (1964) reported the most extensive series of mutations affecting A and B subunits of human LDH. They discovered eight individuals with variants in a population of 940 randomly selected hospital employees in Memphis, Tennessee. The group was composed of 610 Negroes and 330 Caucasians and the frequency of variants was calculated to be 0.9 per cent. Three pedigrees were presented, each showing the appearance of the variant phenotype in three generations. Mutations of either a or b genes therefore appeared to be transmitted in an autosomal codominant manner. Three electrophoretically distinguishable phenotypes, each evidently representing a distinct mutation at the a locus, are illustrated by Kraus and Neely. Any one type may be distinguished from the other two by differences in the electrophoretic

mobility of the hybrid bands. In a given pedigree all the members affected by an a gene mutation exhibited identical electrophoretic mobility of corresponding hybrid bands. The demonstration of three distinguishable types of hybrid isozymes attributable to mutations at the a locus suggests the existence of at least three distinct point mutations of this gene. This implies that each of the three variant a polypeptide chains differs from the normal and from the others by an amino-acid residue. These point mutations must have occurred at portions of the subunit polypeptide chain sufficiently removed from the active site to permit maintenance of normal LDH activity in the variant isozymes. Kraus and Neely found no evidence for linkage of the locus for the B subunit with either the ABH blood group system or the Fy, MNS, Kell and Sutter blood groups.

Neel et al. (1964) were unable to find any departures from the normal erythrocyte isozyme pattern in 79 Brazilian hemolysates examined.

Recently in the light of reawakened interest in the subject a search for variant erythrocyte isozymes was repeated in this laboratory. In the present series 600 Negroes were randomly selected from a patient population in a Washington, D. C. hospital, and 600 white patients were screened from several other hospitals in this area (Vesell, 1965b). Four variants were discovered (Table 1). Three variants involved the A subunit (Fig. 9); in these the electrophoretic mobilities of the corresponding hybrid components were identical. Two of these three variants were kindly typed by Kraus, who found them indistinguishable from those he designated Memphis-1 (Kraus, personal communication). One individual, a 76 year old man, had no living relatives; the second, a 65 year old female, had one daughter, whose erythrocyte LDH isozymes were normal and the third, a 70 year old woman, had a family which was investigated. Six individuals in four generations exhibited the LDH variant. The frequency of variants in this group of 600 Negroes was 0.50 per cent, in the same order of magnitude as that of a comparable group reported by Kraus and Neely (1964). Among the 600 whites studied, only one, a 74 year old unmarried lady with lymphoblastic sarcoma in remission, had a variant pattern. The pattern was unusual in that apparently both b alleles were affected (Fig. 10). Unfortunately no close relatives were available for study. The subject's leukocytes and platelets also revealed isozymic variations identical to those observed in her erythrocytes. The hypothesis of double affection of both b alleles, while satisfactorily accounting for the electrophoretic homogeneity and increased mobility of LDH 1 and LDH 2, fails to explain the apparently normal mobility of LDH 3, the presence of a rapidly migrating sub-band in the LDH 3

TABLE 1—*Variants of Human Erythrocyte LDH Isozymes*

Author	Population Screened	Number of Individuals Studied	Number of Variants Found	Subunit Affected	Frequency of Variants in %
Vesell and Bearn (1962)	Whites, New York	80	0	—	0
	Negroes, New York	280	0	—	0
Boyer (1963)	Whites	50	0	—	0
	American Negroes	300	0	—	0
	Popuans	100	0	—	0
	Nigerians	200	1	B	0.5
Nance et al. (1963)	Brazilians	—	1	A	—
Kraus and Neely (1964)	Negroes, Memphis, Tennessee	610	7	5A, 2B	1.1
	Whites, Memphis, Tennessee	330	1	A	0.3
Neel (1964)	Xavante Indians, Brazil	79	0	—	0
Vesell (1965b)	Negroes, Washington, D. C.	600	3	A	0.50
	Whites, Washington, D. C.	600	1	BB°	0.17
Tashian (personal communication)	Indians (U.S.A.)	284	0	—	0
	Micronesians (Mariana Islands)	238	0	—	0
	Negroes (U.S.A.)	95	1	A	1.05
	Whites (U.S.A.)	28	0	—	0
Davidson et al. (manuscript in preparation)	British	1015	2	A	0.19
	Cypriots, Turkey	245	2	A	0.81
	Nigerians, Ibadan	23	1	A	4.35

°Homozygous.

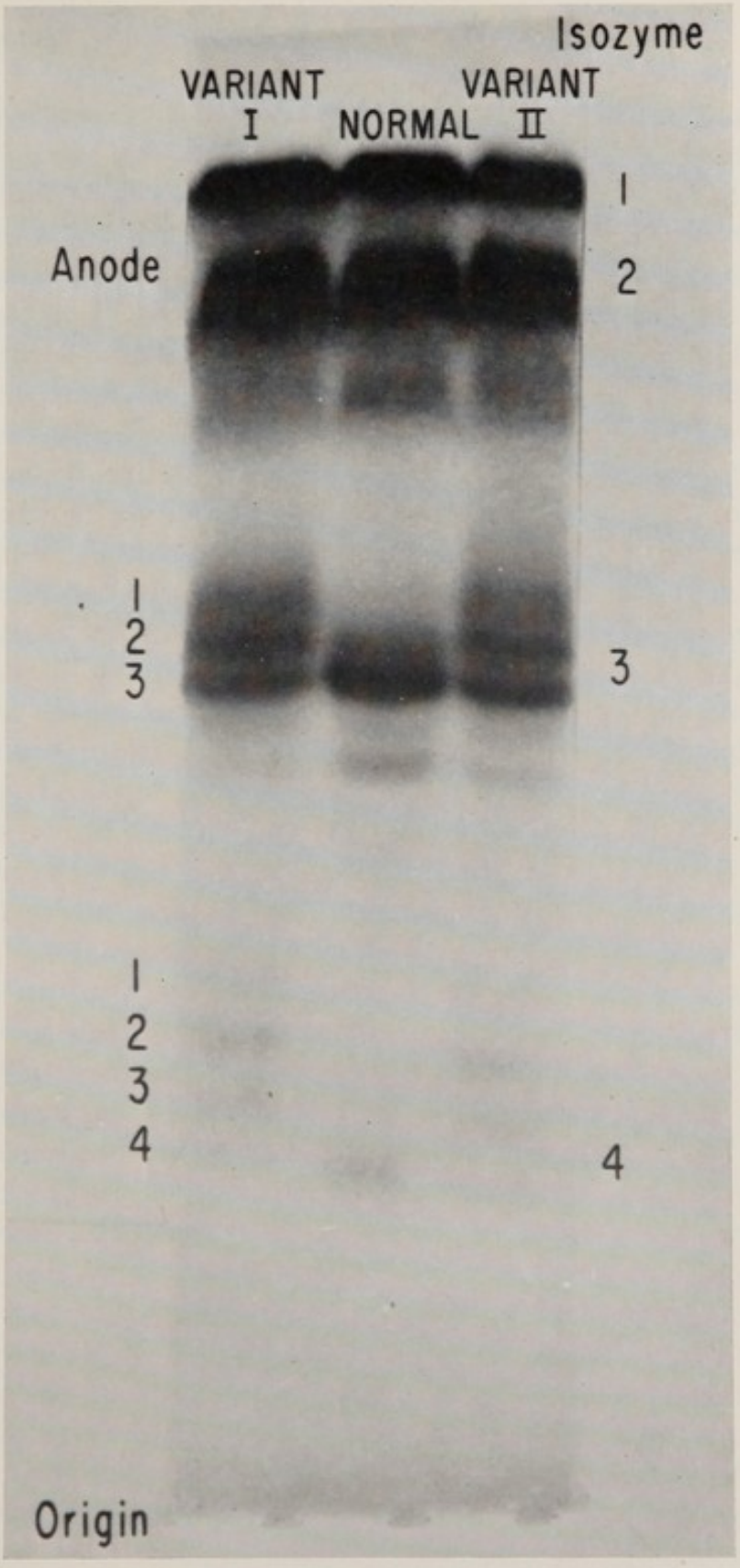

FIG. 9—Starch gel illustrating the two variant isozyme patterns in human erythrocytes found in screening a population of 600 Negro patients in a hospital in Washington, D. C. The normal pattern shown between the variants is that of the only offspring of the individual manifesting variant 1. Both variants occur in the A subunit (from Vesell, 1965b).

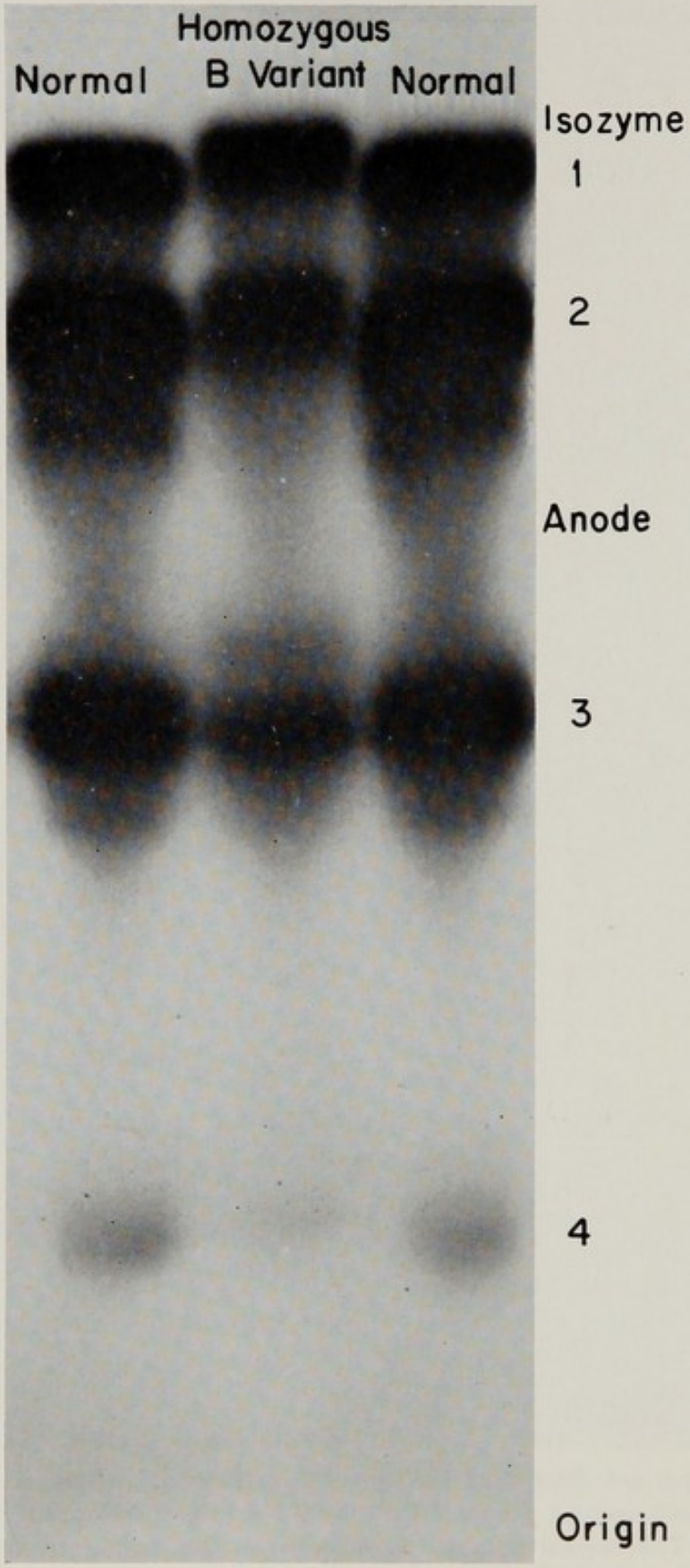

FIG. 10—Photograph of a starch gel illustrating LDH isozymes in erythrocytes from two normal individuals and from an individual with a variant probably produced by affection of both alleles at the b locus.

position, and finally the greater distance between LDH 3 and its more rapidly migrating sub-band than between the normal and variant LDH 1 and the normal and variant LDH 2. Studies on the nature of the B subunit in this variant are required for the elucidation of these problems.

An A variant has been discovered by Tashian (personal communication) in one of 95 Negroes (U. S. A.) screened, giving a frequency of 1.05 per cent. Three other groups, including 284 American Indians, 238 Micronesians from the Mariana Islands and 28 whites (U. S. A.), were studied by Tashian without disclosure of any variants.

These and other data, summarized in Table 1, are unfortunately too few at present to settle the question of whether the frequency of LDH variants differs significantly among populations, although it may eventually be established that variants occur more often in Negroes. In some of the populations screened the frequency of variants exceeds 1 per cent, the figure generally accepted as defining a polymorphism which is a difference among individuals of a high enough frequency so that mutation pressure alone probably could not maintain it. Such a high incidence may occur because, under some circumstances, the variant in the heterozygote form confers a selective advantage, offsetting the deleterious effects exerted by the homozygote. The elimination of genes in homozygotes through decreased fitness (fertility) is balanced by the increased fitness of heterozygous individuals. An example of such a balanced polymorphism in which the heterozygote enjoys a selective advantage is the case of sickle cell hemoglobin in erythrocytes of certain populations living in areas where malaria is hyperendemic (Allison, 1954). Frequencies of variants in the range of 1 per cent, which is much lower than the frequency of the sickle gene in certain areas of Africa, are compatible with the notion that the environmental features which made the mutation adaptively advantageous in previous generations may no longer exist today; the nature of the selection advantage, if any, leading to the LDH polymorphism is unknown. Furthermore, the LDH polymorphism may be essentially neutral. Essentially neutral polymorphisms are becoming increasingly suspected now that so many inherited protein variations, like the LDH polymorphism, are being described, apparently unassociated with disease. Neutral polymorphisms arise through accumulation of mutations over many generations. Since they do not exert sufficiently deleterious effects, neutral mutant genes are not eliminated by natural selection and therefore may attain high frequencies.

In concluding this section on the genetically controlled variants of LDH isozymes it should be restated that a sixth isozyme probably indicates the existence of a third subunit which Blanco and Zinkham

(1963) designated C. Several other examples of mammalian systems exhibiting more than 5 LDH isozymes have been reported by many groups (Allen, 1961a; Philip and Vesell, 1962; Castor and Prince, 1963; Nebel and Conklin, 1964). Although recent structural studies of LDH isozymes discussed in the section on genetic control eliminate the possibility raised by Conklin, Dewey and May (1962) that the five commonly encountered forms of LDH arise by association of a single species of LDH molecule with such substances as NAD, flavoproteins or lipoproteins, some of the additional LDH bands observed on the gel may be accounted for by such association.

Zinkham, Blanco and Kupchyk (1964) have suggested from recombination studies involving pigeon testes as well as from Hardy-Weinberg analysis of six different pigeon populations that LDH synthesis in this species is under the control of three distinct genetic loci: a, b, and c. All pigeons examined were homozygous at the a and b loci, but some were heterozygous at the c locus. Three genotypes were possible at the c locus, cc, cc′ and c′c′. Three phenotypes in the form of testicular LDH isozyme patterns were observed in several different pigeon populations, and the relative frequency of each type in the population analyses agreed with that expected according to the Hardy-Weinberg law for a single pair of alleles. The type I and type III isozyme patterns of pigeon testes exhibited only one and two "X bands," respectively, and were considered to represent homozygous genotypes, whereas there were six X bands in the isozyme pattern of type II. Type II was considered to represent a heterozygous genotype. Evidence for this assumption was obtained from recombination experiments in which a type II isozyme pattern was produced by appropriate mixtures of types I and III followed by freezing in high salt concentrations (Zinkham, Blanco and Kupchyk, 1964).

A formula has been published (Shaw, 1964) for calculating the number of isozymes (i) expected according to the number of different subunits (s) in the isozymic system and the polymer number (p) of each isozyme:

$$i = \frac{(s+p-1)\ !}{p\ !\ (s-1)\ !}$$

From this formula it may be calculated that for polymer number 4, there should be five isozymes when there are two different subunits, 15 isozymes when three distinct subunits exist and 35 isozymes for four distinguishable subunits. LDH in pigeon testes, and presumably also in human testes, having three different subunits and a polymer size

of 4, should theoretically exhibit 15 isozymes. Possibilities to explain why 15 isozymes are not observed on starch gels include (1) overlapping electrophoretic mobilities of some bands and/or (2) certain *in vivo* epigenetic factors preventing the formation of some of the isozymes from the available subunits and/or (3) insufficient concentrations of one or several of the subunits to permit the production of all the theoretically expected isozymes.

Ontogenetic Studies of LDH Isozymes

Before the development of the subunit theory of LDH isozyme structure, embryologists sought clues to the adult zymograms by comparing them to those existing at various stages of an organism's development (Markert and Møller, 1959; Flexner et al., 1960). Markert and Møller (1959) first demonstrated such differences by contrasting the zymograms of a variety of adult and fetal pig tissues. In most cases the fetal tissue differed in isozyme pattern from the adult tissue. Flexner et al. (1960) described in developing rat brain cortex serial changes consisting of progressive increases in anionic isozymes with maturation. Sequential alterations in the zymogram of chicken breast muscle from predominance of anodal bands early in development to predominance of cathodal bands at later stages were reported 3 years later (Cahn et al., 1962; Philip and Vesell, 1962). In chick liver there occurred during development sequential alterations in the zymogram similar to those observed in chick muscle, whereas the changes in chick heart were less dramatic and toward dominance of anodal rather than cathodal bands (Philip and Vesell, 1962). These observations are illustrated in Figure 11. Gradual sequential alterations in the LDH isozymes of chick tissues during development have been reported by Lindsay (1963). It should be emphasized that the patterns diagrammed in Figure 11 apply at the concentrations indicated, but that at other concentrations differences occur. If the concentration of LDH activity in the extracts of 8-day muscle and liver is raised to approximately 100,000 units/ml. then cathodal bands may be seen faintly on the starch gel. Thus all isozymes are present even at the earliest embryonic stages and developmental alterations are characterized by marked shifts in the distribution of total activity among the bands, but not in appearance of bands previously absent. From a genetic point of view this suggests that genes directly concerned with producing the LDH isozymes are all operative from very early stages of development rather than, as suggested by Cahn et al. (1962), being switched on at different times. Markert and Ursprung

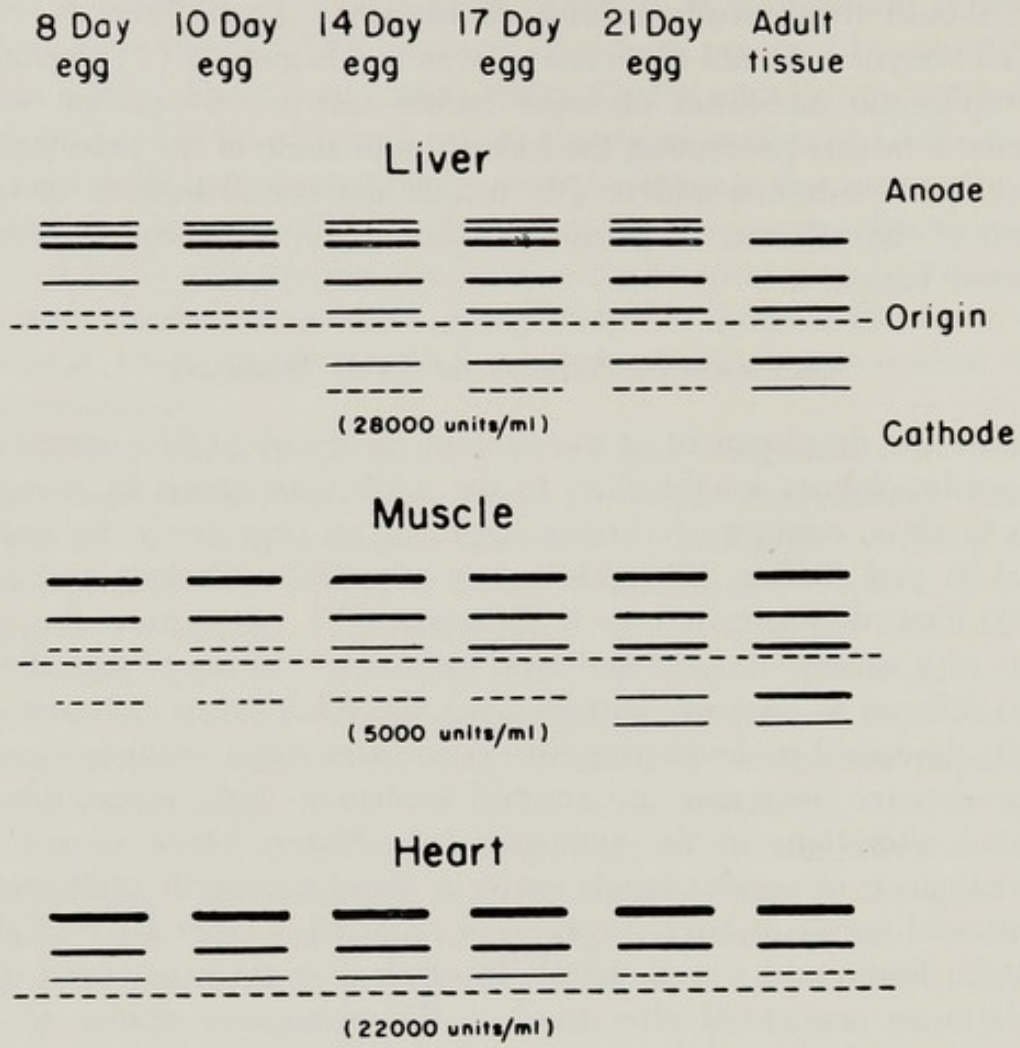

FIG. 11—Diagram of the intensity and localization of LDH isozymes from chick tissues obtained at various days after fertilization and separated by starch gel electrophoresis (from Philip and Vesell, 1962).

(1962), studying the ontogeny of mouse tissues, described prominence of LDH 5 in all early embryonic cells, as opposed to the prominence of LDH 1 in embryonic chick tissues, and reported in diaphragm and heart increased intensity of anodal isozymes with development. Rabbit tissues correspond more to chick tissues in ontogeny than to mouse tissues (Vesell, Philip and Bearn, 1962b). During ontogeny human tissues undergo changes similar to those in rabbit and chick (Vesell, Philip and Bearn, 1962b, Wiggert and Villee, 1962).

During ontogeny sequential alterations in isozyme patterns may be interpreted either as reflecting changes in the relative activities at the a and b genetic loci or as indicating changes originating at the level of ribosomal production of A and B subunits. The specific genetic and epigenetic factors operative during development that induce differential predominance of either the A or B subunits remain unknown.

Phylogenetic Studies of LDH Isozymes

More than 10 years ago comparisons of lactic dehydrogenases derived from various species were reported; kinetic as well as immunological techniques revealed differences between the LDHs of *Schistosoma mansoni* and rabbit muscle (Mansour and Bueding, 1953; Henion, Mansour and Bueding, 1955). Kaplan et al. (1960) effectively employed NAD analogues to display various phylogenetic differences and suggested that these methods might be valuable in studying the interrelationship and origin of species. Markert and Møller (1959) demonstrated that starch gel electrophoresis could reveal very subtle differences in the isozyme patterns of heart extracts from five species. In an investigation of the erythrocyte isozymes from 18 species, including representatives of all five classes of vertebrates, more than 40 electrophoretically distinguishable LDH isozymes were visualized on starch gels (Vesell and Bearn, 1962b). Figure 12 shows that phylogenically closely related species generally exhibit similar patterns, whereas more distantly related species tend to display differences in LDH pattern. Figure 12 also reveals exceptions to this principle as indicated by the almost identical patterns of human and guinea pig isozymes.

The possibility that chromosomal duplication could account for the development of isozymes has been raised. According to this hypothesis, early in the phylogenetic scale of evolution multiple forms LDH arose from a single enzyme by fragmentation during crossing-over of chromosomes. The piece of genetic material broken off during crossing-over would have been incorporated later in the chromosome. The repeated gene initially would yield polypeptides identical to that produced by the parent gene, but a mutational event affecting either the repeated gene or parent gene would alter the polypeptide formed by that gene. The altered polypeptide might differ from the polypeptide produced by the gene not undergoing mutation in just such ways as the A and B subunits of LDH differ. This theory predicts the occurrence of organisms containing LDH composed of a single type of subunit and also the existence of a precise point in the phylogenetic scale where two subunits arose. Fulfilling the first prediction, Yoshida (1965) isolated an LDH from *B. subtilis* composed of 4 identical subunits, each of 36,000 molecular weight.

Finally a number of recent studies on the comparative enzymology of LDH by Kaplan and his associates with extensive chemical data should be mentioned (Fine, Kaplan and Kuftinec, 1963, Fondy et al., 1964, Pesce et al., 1964; Wilson et al., 1964). These studies were directed toward demonstrating that the "hybrid" isozymes 2, 3 and 4 are inter-

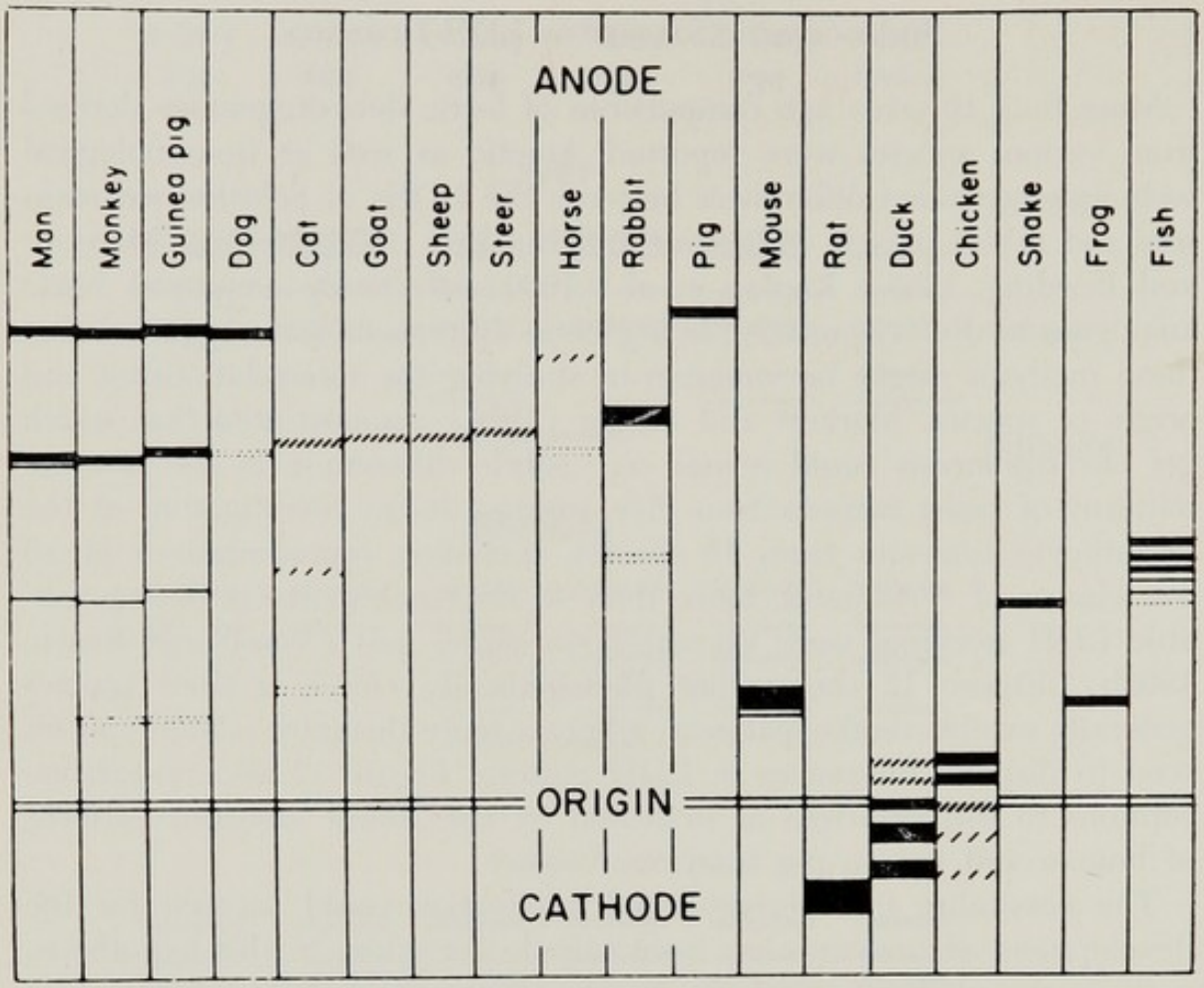

FIG. 12—Diagram of the intensity, size and localization of LDH isozymes from erythrocytes of 18 vertebrate species separated by starch gel electrophoresis (from Vesell and Bearn, 1962b).

mediate in chemical structure and kinetic behavior between the extremes of the "pure types" 1 and 5. Also Kaplan's group attempted to establish a phylogenetic scale of evolution based on chemical differences among the LDHs of various species. Kaplan and associates, electing to avoid the term isozyme, refer to LDH 1 and LDH 5 as pure "heart" and "muscle" types respectively and to LDH 2, 3 and 4 as "hybrids." Because of the numerous contributions to the literature by Kaplan's group, this nomenclature has enjoyed much popularity, but it is somewhat misleading since neither heart nor muscle from most vertebrate species is homogeneous, as the unwary reader may suspect from the word "pure," but each contains several isozymes; nor in any vertebrate system thus far studied is the electrophoretic pattern of heart or muscle unique. For example, Figure 1 clearly demonstrates that human skin and liver have isozyme patterns indistinguishable from that of muscle. Furthermore, studies during ontogeny reveal that the sequential alterations in chick, rabbit and human muscle are also not

unique but resemble those observed in liver. The terms "heart" and "muscle" type were developed before Kaplan and his group utilized techniques for separating the isozymes which they previously had subjected to kinetic studies with NAD analogues in unresolved mixtures (Kaplan et al., 1960). The use of the word hybrid when restricted to LDH 2, 3 and 4 is also somewhat misleading because it implies the existence of a different synthetic mechanism for the construction of isozymes 2, 3 and 4 than obtains for the "pure muscle and heart" types, whereas recombination experiments suggest that all the isozymes may be constructed by a similar process, random recombination of subunits. True hybridization has been accomplished by subunit recombination of isozymes prepared from two different species (Markert, 1965a, b). In these hybridization experiments isozymes of different mobility from either parent form are produced, indicating differences between the two species in the structure of their subunits (Fig. 13).

Environmental Conditions Producing Alterations in Tissue Isozyme Patterns

In the section on genetic control (part A), experiments were described in which the pattern of isozymes resulting from recombination of LDH 1 and LDH 5 remained unchanged despite the addition of various substrates and coenzymes. These reagents apparently exerted no effect *in vitro* on the recombination of subunits under the conditions employed. However, certain alterations in the cellular environment have been effective in causing shifts of the isozyme pattern. The environments of cells in tissue culture, of cells undergoing malignant proliferation and of cells in certain tissues exposed to hormonal stimulation exhibit such alterations.

Embryonic chick, rabbit and human cells from a variety of tissues when grown *in vitro* with Eagle's medium and 10 per cent calf serum exhibit shifts in isozyme pattern (Philip and Vesell, 1962; Vesell, Philip and Bearn, 1962b; German et al., 1964). In culture under the conditions described in these papers all the tissues of a given organism assume a typical uniform pattern, characterized by prominent cathodal bands and diminishing or disappearing anodal bands. This observation is illustrated in Figure 14 which shows the regularity of the pattern displayed by a variety of human tissues in culture over differing periods. Each tissue exhibits a pattern characterized by a heavy cathodal band and lighter anodal bands. Earlier Tsou (1960) had shown that isozymes of several different enzymes existed in cells grown in culture.

Alterations in isozyme patterns of cells in culture may be interpreted

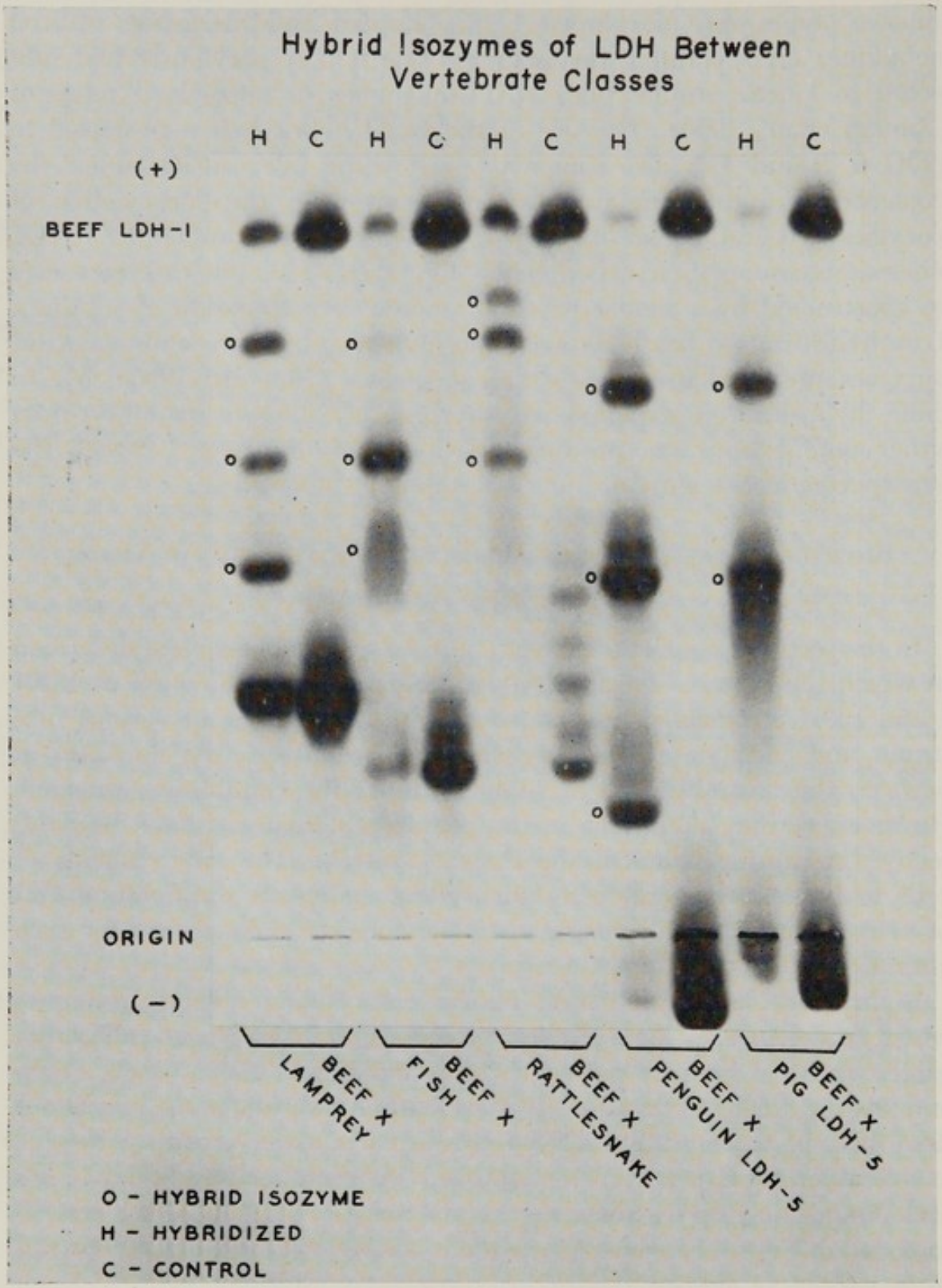

FIG. 13—Photograph of a starch gel illustrating hybrid isozymes produced by mixing beef LDH 1 with LDHs isolated from various vertebrates and by freezing the mixture in 1 M NaCl (from Markert, 1965a).

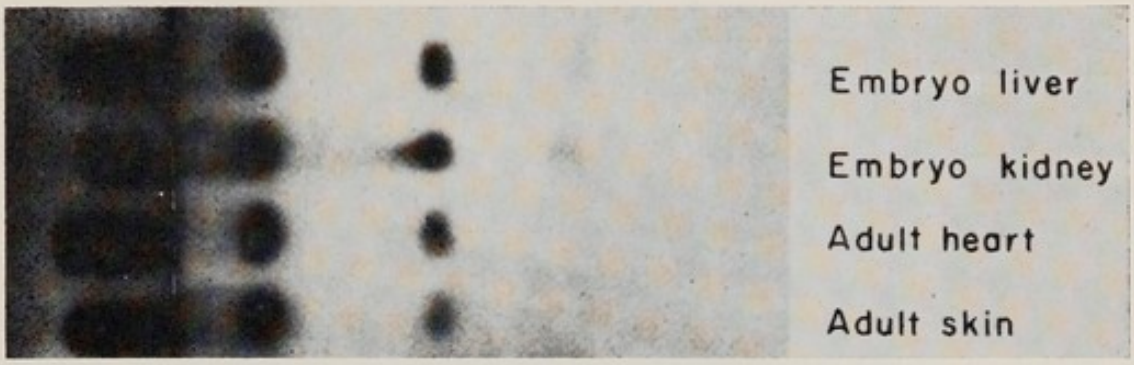

FIG. 14—Photograph of a starch gel illustrating the LDH isozyme pattern of various human tissues in culture. Each sample contained 10,000 units/ml. Embryo liver was in culture 82 days; embryo kidney, 58 days; heart, 91 days; skin, 112 days (from Vesell et al., 1962b).

in the light of the hypothesis for the genetic control of the LDH subunits and its corollary that tissues maintain different isozyme patterns *in vivo* because of epigenetic conditions leading to relative dominance of either the a or b genetic loci. In culture, cells from different environments are all exposed to identical conditions, and they respond in identical fashion by developing a uniform isozyme pattern, a pattern suggesting predominance of the a over the b genetic locus. The rapid rate of change in isozyme pattern of chick embryo muscle cells when grown in culture is illustrated in Figure 15.

Possible explanations of the isozyme alterations *in vitro* include dedifferentiation or modulation under the simplified cultural conditions or outgrowth of a particular cell population favored by the *in vitro* environment. The latter possibility clearly obviates the necessity of a genetic explanation for the shift in isozyme pattern.

The pattern that emerges in culture is one of predominance of LDH 5, the isozyme most resistant to anaerobic conditions and high lactate concentrations. Hence the shift toward LDH 5 may be related to the anaerobic environment prevailing in culture. By raising the oxygen tension of cells grown in culture Cahn (1964) prevented the shift toward LDH 5. Several other recent studies have appeared concerning the isozyme patterns of cells grown in tissue culture. In certain long-term cultures Nitowsky and Scderman (1964) were able to show retention of the tissue isozyme patterns, although most of their other cells in culture exhibited predominance of LDH 5. One report described alterations in isozyme pattern of cells grown in chemically defined culture media without serum effected by the addition of either 10 per cent horse or 10 per cent calf serum for 5 days (German et al., 1964).

The LDH isozymes in sera from patients with leukemia were demon-

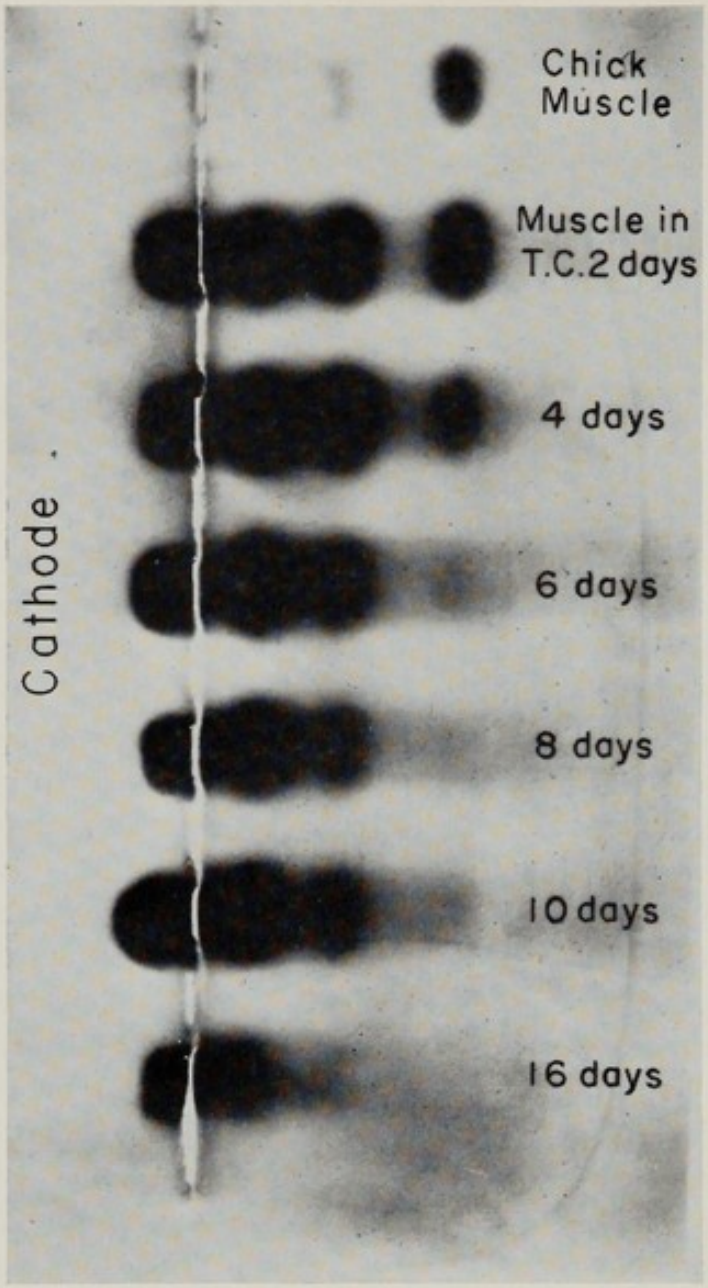

FIG. 15—Photograph of a starch gel showing sequential alterations in LDH isozymes of 10-day chick muscle after trypsinization and in tissue culture for 2 to 16 days. All tissue culture extracts were applied to the gel at approximately 20,000 units of LDH activity/ml. (from Vesell and Philip, 1963).

strated to have increased amounts of LDH 2 (Vesell and Bearn, 1957). Starkweather and Schoch (1962) described differential elevations of LDH 3 in malignant tissues. They also observed alterations in the kinetic behavior of LDH 3 in malignant tissues. Zondag (1964) also reported elevations of the "middle LDH isozymes" in plasma from certain individuals with cancer, but in addition described cases of testicular seminoma, testicular teratoma and ovarian dysgerminoma in which the tumor tissue exhibited elevations of LDH 1 and LDH 2. In contradiction to these

observations are those of Gerhardt et al. (1963) and Goldman, Kaplan and Hall (1964). The former group described elevation of LDH 5 in malignant brain tumors; the latter group reported a consistent shift in cancer tissue toward LDH 5 in a study of 400 normal and neoplastic human tissues of a variety of malignant grades. Goldman, Kaplan and Hall (1964) interpreted their observations to indicate that prevalence of anaerobic conditions in tumor tissues might cause predominance of LDH 5, the isozyme most resistant to high lactate concentrations. Zuppinger, Richterich and Rossi (1962) examined the serum isozymes in patients with various tumors but observed no consistent pattern.

Hormonal stimulation has been shown to play an important role in maintaining the integrity of the isozyme pattern in certain tissues. In mammary gland from ovariectomized mice the isozyme pattern is markedly different from that of lactating mammary gland in normal mice. Allen (1961a) also described striking changes in the isozyme pattern of the uterus from ovariectomized mice following treatment with estradiol. The mouse epididymal pattern following section of the vasa efferentia was characterized by loss of one of the isozymes, but retention of the others (Allen, 1961a). In 1963 Richterich et al. discovered that human uterus during pregnancy exhibited a shift in isozyme pattern toward LDH 5 and suggested that normal human uterus acted like other visceral muscles, displaying an isozyme pattern similar to them, but that pregnant uterus resembled skeletal muscle in both function and isozyme pattern. Based on these observations Dawson et al. (1964) and Goodfriend and Kaplan (1964) administered estradiol to immature rats and rabbits and were able to confirm the report of Richterich, Schafroth and Aebi (1963). Administration of estradiol stimulated the synthesis of A subunits. With cessation of hormone, the concentration of A subunits fell and the proportion of A to B subunits returned to normal values (Fig. 16). These observations were interpreted as indicating direct action of the hormone estradiol at the a and b genetic loci with differential stimulation of the a gene. This conclusion was drawn because puromycin administration following hormone treatment resulted in a proportionate decline in the concentrations of A and B subunits and because preferential stimulation of the synthesis of A subunits could be blocked by prior administration of Actinomycin D (Goodfriend and Kaplan, 1964). Testosterone and progesterone, however, resulted in a proportional increase of both subunits A and B.

Finally, Allison, Gerszten and Sanchez (1964) reported that rabbits receiving triiodothyronine exhibited decreased total hepatic LDH activity, with complete loss of LDH 5 and a decline in LDH 2, 3 and 4. In

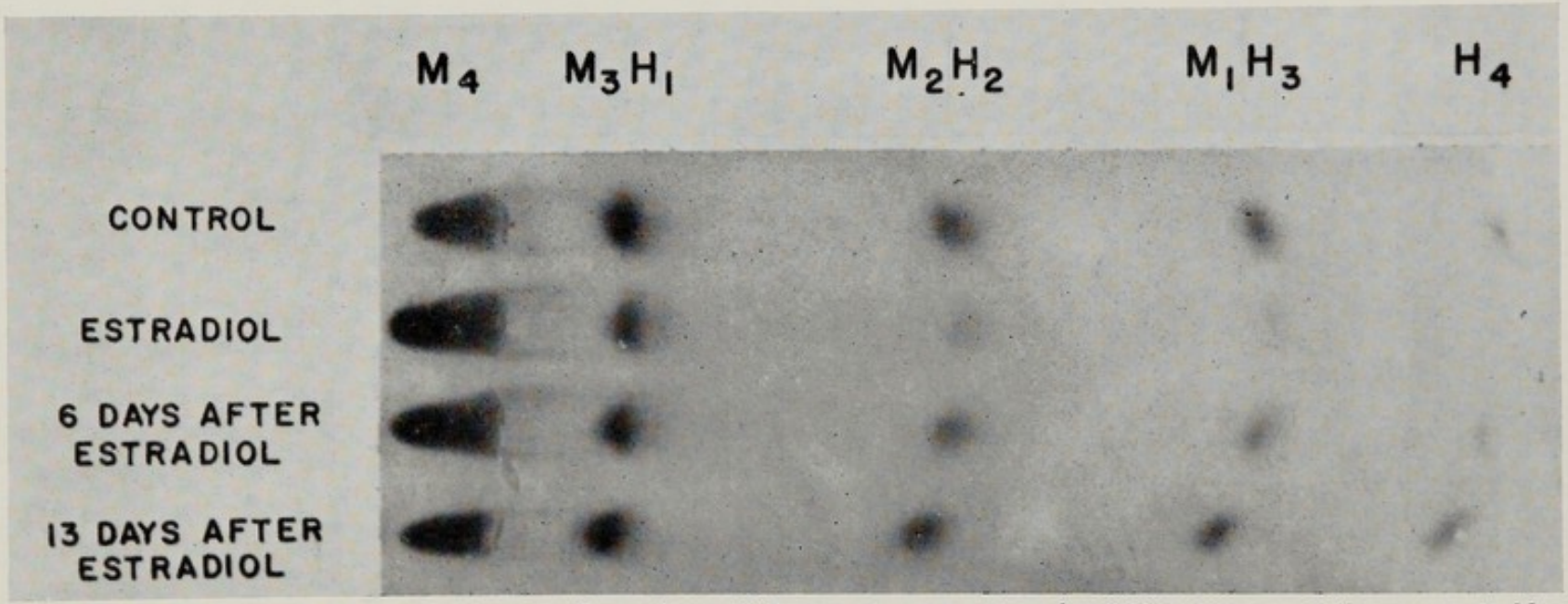

FIG. 16—Photograph of a starch gel showing effect of estradiol administration on the LDH isozymes of rat uteri. Note decreased intensity of anodal isozymes and increased intensity of "M_4" (from Goodfriend and Kaplan, 1964).

these experiments the observation of differential loss of LDH 5 following hormone administration may be partially attributable to the dilution effect in which LDH 5 is more subject to activity loss than LDH 1 and LDH 2.

Subcellular Localization of LDH Isozymes

One of the earliest theories advanced to explain the existence of multiple proteins all performing similar functions was that some LDH isozymes might meet better than others specific metabolic requirements of certain subcellular particles (Vesell and Bearn, 1961; Allen, 1961b). Subsequently, evidence has developed to support the hypothesis of differential intracellular localization of isozymes and these data will now be reviewed. Previously, it was mentioned that the mature human erythrocyte and the human platelet (Fig. 2) were unique both in the absence of a nucleus and in lacking LDH 5 on starch gel electrophoresis. Another piece of evidence linking LDH 5 with the nucleus was derived from the comparative study of erythrocyte LDH isozymes of vertebrates; only those species having a nucleated erythrocyte in the peripheral circulation exhibited prominent cathodal isozymes, as shown in Figure 12. Several additional relationships between LDH 5 and the nucleus were obtained (Vesell and Bearn, 1962a): (1) LDH 5 could not be visualized by starch gel electrophoresis in normal circulating guinea pig erythrocytes, whose isozyme pattern resembled that of human erythrocytes. But LDH 5 appeared in guinea pig hemolysates following reticulocytosis induced by phenylhydrazine administration; (2) in 500 humans no LDH 5 was observed in circulating erythrocytes, but LDH 5 was discovered in hemolysates from four individuals with reticulocytosis; (3) in nuclei isolated from duck erythrocytes and disrupted by ultrasonic vibration, LDH 4 and LDH 5 were very prominant in contrast to the non nuclear fraction which exhibited mainly LDH 1, 2 and 3.

Allen (1961b) reported differential localization of LDH isozymes of rat kidney to various intracellular compartments. With the use of various substrates Allen (1961a) demonstrated that in mouse epididymis, LDH activity could be localized to different cellular regions. Brody and Engel (1963) made use of the fact that urea differentially suppressed LDH 5, whereas lactate in high concentrations differentially suppressed LDH 1 and LDH 2. They examined sections of normal human skeletal muscle incubated with either urea or high lactate and discovered that the sites of enzyme localization were not uniform. Slow-moving LDH isozymes resided primarily in the network of type I fibers and in the stellate formations; fast-moving isozymes predominated in the network of the

type II fibers. Blanchaer and Van Wijhe had previously (1962) reported differences in the LDH isozyme patterns of red and white skeletal muscle from the guinea pig, rabbit and mouse; red muscle fibers exhibited all five bands, white ones only LDH 4 and LDH 5. They had anticipated that red muscle would have predominantly LDH 1 and LDH 2, but were unable to prepare red muscle free from white muscle contamination. However, their expectations were fulfilled by Fahimi and Amarasingham (1964), who stated that white muscle fibers contain predominantly slower moving LDH isozymes, whereas red muscle fibers exhibit the more rapidly migrating ones. Smith and Kissane (1963), employing only kinetic reactivities with two different pyruvate concentrations, demonstrated different distributions of LDH isozymes in various portions of adult rat kidney. Cortical tubules exhibited mainly the reactivities characteristic of LDH 1 and LDH 2, whereas the papilla revealed reactivities characteristic of LDH 4 and LDH 5.

Finally, experiments performed in this laboratory demonstrated differences in the subcellular localization of LDH isozymes from adult rat kidneys. Kidneys obtained from adult Sprague-Dawley rats were washed several times in cold 0.25M sucrose solution containing 0.05M Tris buffer pH 8.6 and 10^{-4}M $MgCl_2$. The kidneys were then homogenized, one part tissue to five parts solution, in a Potter-Elvehjem homogenizer with motor driven pestle at 4°C. Fractionation into nuclear, mitochondrial and microsomal portions was accomplished by differential centrifugation (Hogeboom, 1955). The microsomal preparation was treated in some experiments with deoxycholate to yield a ribosomal fraction (Wettstein, Staehelin and Noll, 1963). The isozyme patterns of whole homogenate disrupted by ultrasonic vibration were compared with the patterns of ultrasonically disrupted nuclei, mitochondria, microsomes and ribosomes. The results are illustrated in Figure 17. Compared to the pattern of whole homogenate, the nuclei exhibited increased LDH 5 activity, whereas the mitochondrial and ribosomal preparations contained mainly isozymes 1 to 4. Different results were obtained by Keck and Choules (1962) who also examined LDH isozymes of rat kidney and liver and described increased LDH 5 in ribosomes. They ascribed elevated LDH 5 activity of ribosomes to binding of basic isozymes (LDH 4 and LDH 5) by ribonucleoproteins. However, in experiments such as illustrated in Figure 17, no LDH 5 was observed either in ribosomes or in microsomes prepared from rat kidney.

The LDH isozymes are highly soluble and probably "leak over" during preparative procedures from one fraction into another. These procedures involve many variables slight alterations of which can produce significant differences in the isozyme patterns of the particles obtained.

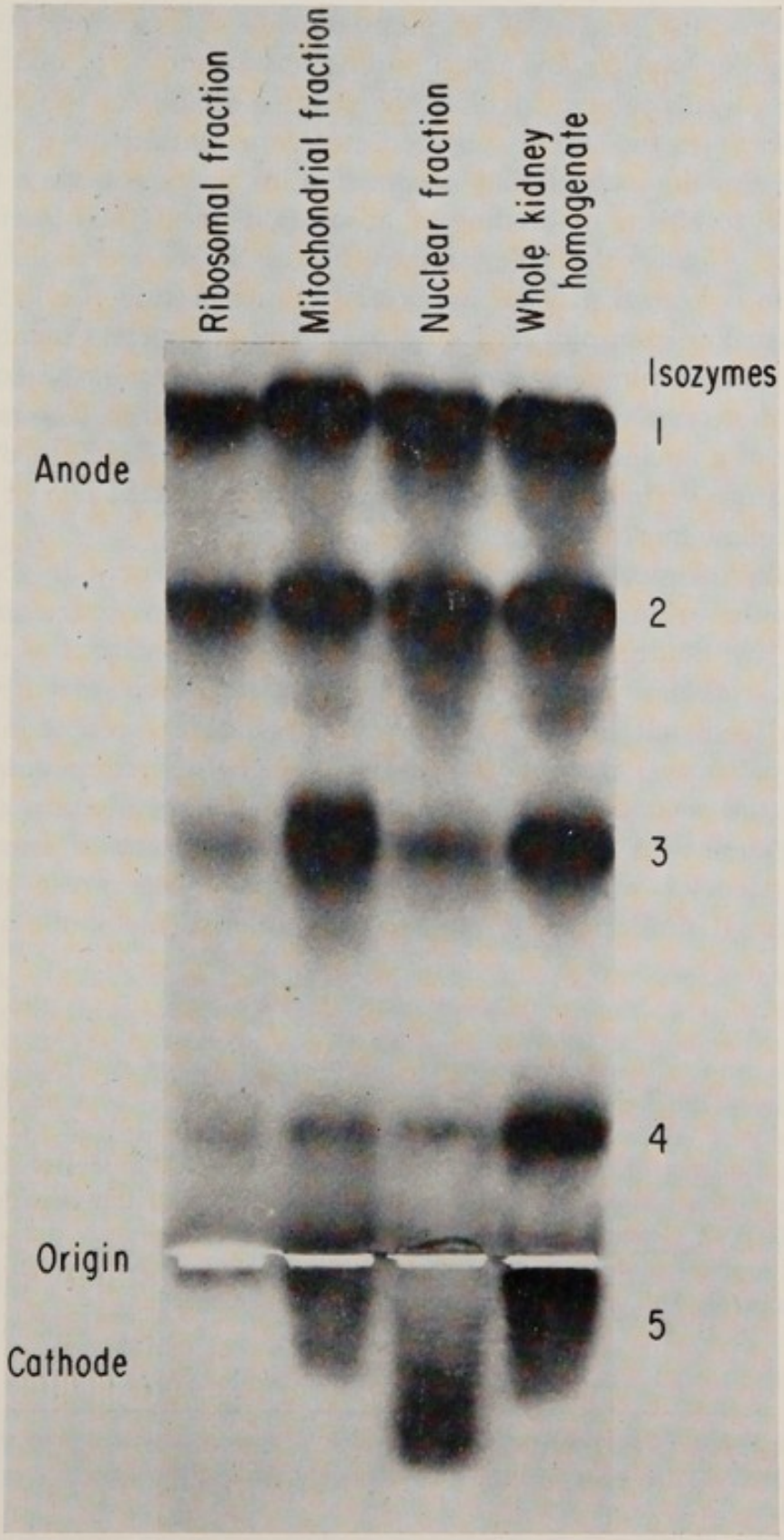

FIG. 17—Photograph of a starch gel showing LDH isozyme patterns in subcellular fractions of rat kidney. Homogenates of whole kidney, nuclei, and mitochondria prior to application on the gel each contained 16,000 units/ml.; the ribosomal preparation contained 10,000 units/ml.

Assessment of the degree of contamination of the isozyme pattern in one subcellular particle by contributions from another is difficult. Another unresolved problem involves the relative affinity of the differently charged isozymes for various charged structures within the cell; certain differences in the subcellular localization of isozymes may thus be attributable to effects of binding or adsorption during the fractionation procedures. Finally, the role of intracellular LDH metabolism about which little is known may be important in interpreting the localization experiments. For example, according to several parameters mentioned in the section on the physicochemical properties of isozymes, the activity of LDH 5 is more easily lost than that of the other isozymes. The metabolic machinery of a nucleated cell may tend to preserve LDH 5; in the platelet and mature vertebrate erythrocyte accelerated catabolism of LDH 5 may thus follow loss of the nucleus.

What physiological role specific isozymes could serve by being associated with various subcellular particles also remains speculative; but, as shown by Stadtman, Cohen and LeBras (1961) for the existence of two aspartokinase isozymes in *Escherichia coli,* each isozyme may be subject to feedback control by different metabolites present in two alternative pathways. One of these pathways might predominate in one organelle, the second pathway might control the metabolism of a different organelle. In any event subcellular localization of isozymes to different particles would permit them, despite their great structural and functional similarities, to engage in distinctive metabolic activities.

Acknowledgments

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CHAPTER 6

Serum Lipoprotein Allotypes in Man

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DURING THE PAST DECADE it has become apparent that human beings differ in their plasma proteins as well as in their blood cells. Genetically controlled variations that are common or polymorphic have been described in haptoglobins and transferrins (Smithies and Connell, 1959), γ-globulins (Grubb, 1957), the Gc proteins recognized by immunoelectrophoresis (Hirschfeld, 1959) and in plasma alkaline phosphatases (Boyer, 1961; Arfors, Beckman and Lundin, 1963). Somewhat less common variations are found in plasma pseudocholinesterases (Kalow, 1959), and many rare inherited variations in human plasma proteins have been described (see Allison, 1964a; Blumberg 1964).

Although the known chemical differences between the alternative types of human plasma proteins appear to be small (see Smithies, Connell and Dixon, 1962, for haptoglobins), these can be sufficient to allow the types to be distinguished by immunological tests with animal antisera (Korngold, 1963). The possibility therefore existed that alternative forms of these or other plasma proteins might be sufficiently different in structure to induce plasma-protein isoimmunization when blood is transfused from one human subject to another.

Attempts to demonstrate individual differences in serum proteins within a species by immunological techniques have a long history, which has recently been reviewed by Hirschfeld (1963). Differences between the reactions of individual human sera with antisera prepared in rab-

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bits or other animals were reported by Weichardt (1906), Bruck (1907), Fischer and Raquet (1930), Smith (1939) Cumley and Irwin (1943), Hess and Butler (1962) and Alepa and Steinberg (1964). Others however, were unable to repeat these observations (Marshall and Taegue, 1908; Fitzgerald, 1909; Goldberg and de Gara, 1948). This does not necessarily mean that the original observations were incorrect, because antisera themselves vary to a considerable extent, and only some of them might be suitable for revealing individual differences.

Another approach was provided by isoimmunization, that is, injecting serum from one animal into another of the same species. In this way it is possible to avoid formation of species-specific antibodies, which usually predominate over and obscure reactions dependent on individual differences. Any isoantibodies then formed might be expected to show individual variation. Such experiments were pioneered by Schütze (1902), who found that pooled serum from several rabbits injected into another rabbit gave rise to a precipitating antibody that reacted strongly with 2 out of 32 individual rabbit sera tested and gave no reaction with the other sera. Oudin (1956) immunized rabbits with sera from other rabbits and obtained isoantibodies which gave well-defined precipitation reactions in agar gels. Detailed studies of the inheritance of the antigens defined in this way has shown that they are determined by independent genetic loci affecting γ-globulin synthesis (Dray et al., 1962). The term "allotypes" was introduced by Oudin for such individual variations within a species. Although the word means "alternative types," and could be applied to variations of any kind, it is often used in a more restricted sense to refer to differences demonstrable by isoantibodies.

Evidence of Serum Protein Isoimmunization in Man

Marie (1916) reported that 2 of 21 patients after intravenous injection of human serum formed precipitins reacting with the serum from the donor as well as with serum from certain individuals. Although some controls were included, the reader is referred to the original paper in assessing the validity of Marie's claim that his results demonstrate individual specificity in human sera. György and Witebsky (1929) described a child who had received blood from several donors, including the father. The child's serum did not agglutinate the red blood cells of any donors, but fixed complement when mixed with the serum of the father but not with serum from the mother. It was concluded that the child's serum contained an antibody directed against a constituent in the serum of the father.

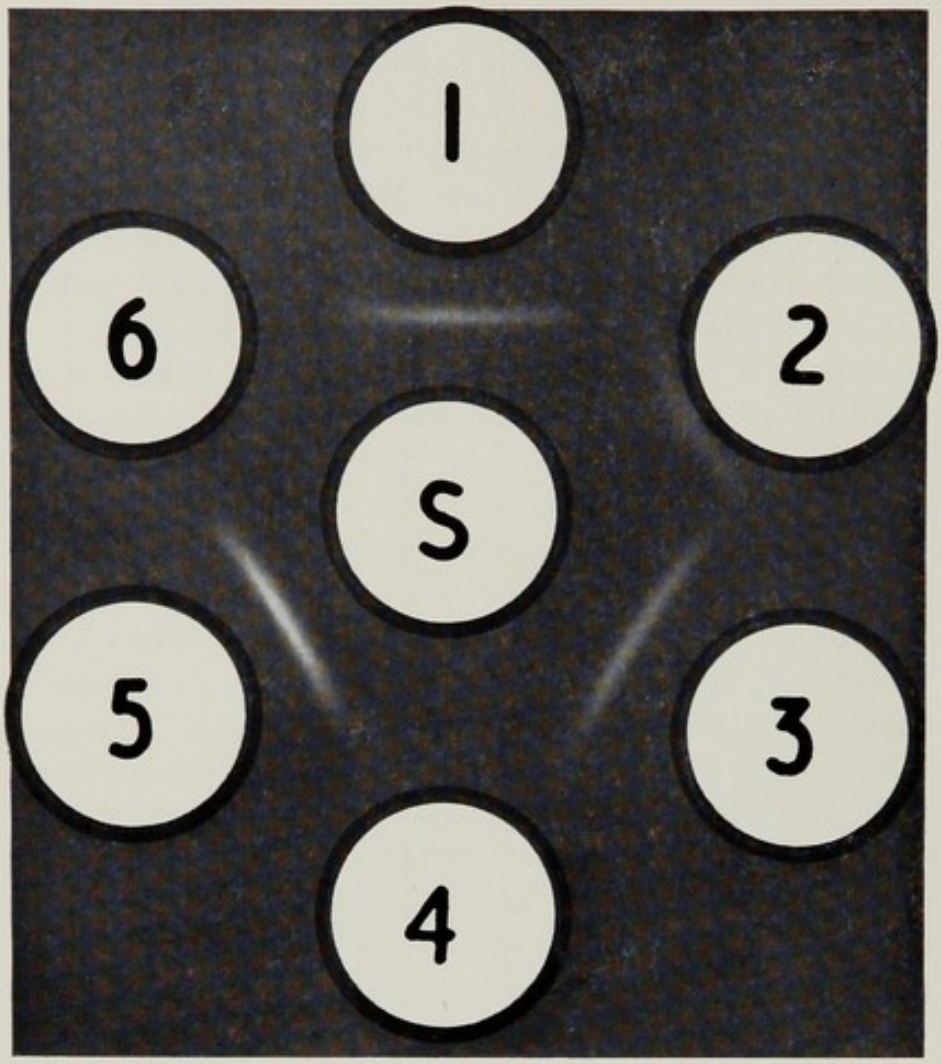

FIG. 1—The precipitation reaction in an agar-gel Ouchterlony plate between the serum of a multiply transfused patient (C. de B.) and some, but not all human sera from a panel of normal subjects. Numbers 1, 3 and 5 are strong reactors, number 2 is a weak reactor and numbers 4 and 6 are nonreactors (Allison and Blumberg, 1961).

Allison and Blumberg (1961) found that the serum of a patient (C. de B.) who had received multiple transfusions gave well-defined precipitation reactions in agar with some, but not all, individual sera from a panel tested (Fig. 1). Family studies showed that the formation of an antigen reacting with the serum of the transfused subject was inherited independently of other known systems, and thus a new human serum protein polymorphism was revealed. Since an antigenic component was involved, but the exact protein was not then known, the name Ag was proposed for the system.

Characterization of the Antibody

Serum from transfused patient C. de B. was submitted to immunoelectrophoresis in agar gel, and a serum containing precipitating antigen

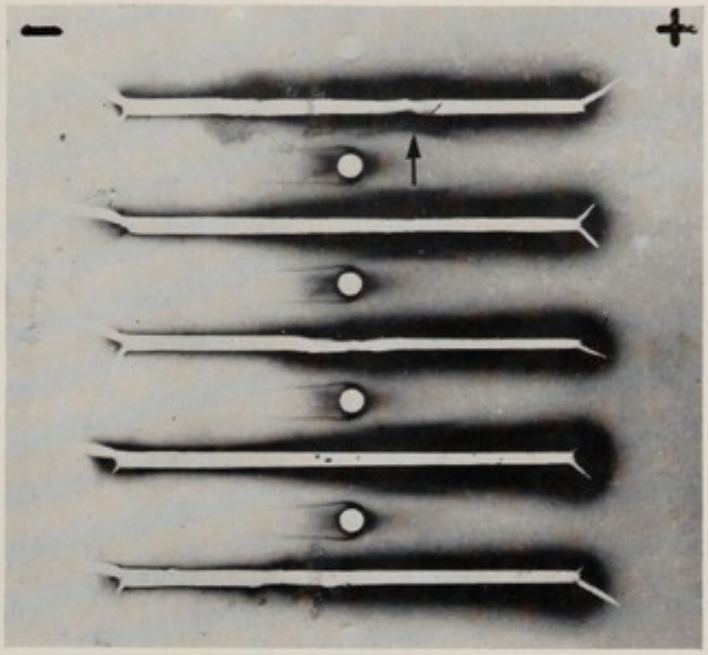

FIG. 2—Agar-gel immunoelectrophoresis to characterize the antibody in serum C. de B. The serum of C. de B. was placed in the circular wells and submitted to electrophoresis, and a serum containing the precipitating antigen, concentrated three times, was placed in the rectangular well. A broad precipitin line (arrow) is seen in the position of the γ-globulin (Allison and Blumberg, 1961).

was placed in the side well. Within 18 hours a well-defined precipitation line appeared in the γ-globulin region (Fig. 2). This result, together with the shape of the precipitation curve in the Ouchterlony plate (convex towards the antibody well) suggested that the antibody was a relatively low molecular weight γ-globulin. This interpretation was confirmed in an experiment in which the 7 S- and 19 S-γ-globulin components in the patient's serum were separated by sucrose density-gradient centrifugation (Allison and Blumberg, 1961). Precipitation lines in Ouchterlony plates were obtained with low molecular weight fractions giving no reaction with specific immune rabbit serum against 19 S-γ-globulin as described by Franklin (1960) and Allison and Blumberg (1961). It has also been found (Allison, unpublished) that the antiserum (C. de B). withstands concentrations of mercaptoethanol sufficient to inactivate 19 S antibody. It can therefore be concluded that the antibody is a 7 S-γ-globulin, or γ G in current terminology.

Characterization of the Antigen

One of the panel sera containing the antigen was concentrated by ultrafiltration and submitted to immunoelectrophoresis. The antibody well was filled with serum from the transfused patient C. de B. A well-defined precipitation line developed in the α_2 region (Fig. 3) (Allison

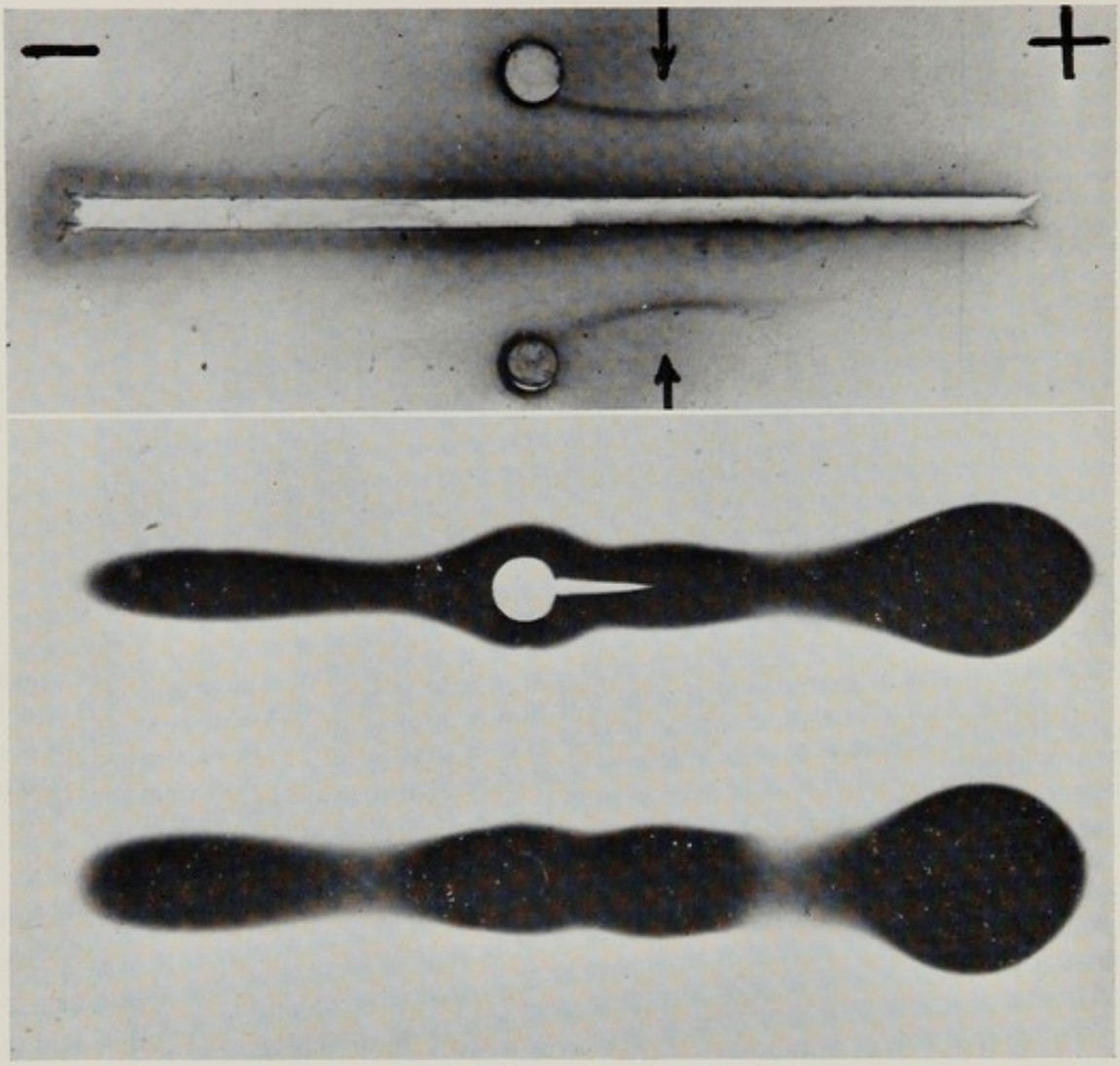

FIG. 3—Agar-gel immunoelectrophoresis to characterize the antigen. The serum of a reactor was concentrated three times by ultrafiltration and submitted to electrophoresis. The serum of patient C. de B. was placed in the rectangular antibody well. The position of the precipitin line (arrow) shows that the antigen has in agar the mobility of an α_2-globulin. Above, unstained precipitin lines; below, protein stain (Allison and Blumberg, 1961).

and Blumberg, 1961). The antigen was later identified as a low density β-lipoprotein, which migrates as an α_2-globulin in agar-gel electrophoresis but as a β-globulin on paper electrophoresis. Fractions of β-lipoproteins of density less than 1.063, obtained by flotation on concentrated salt solutions in an ultracentrifuge, were shown to contain the antigen reacting with antiserum C. de B. The precipitation line was colored with the lipid stain Sudan Black B and was continuous with that produced in whole serum by rabbit antibody against human β-lipoprotein (Blumberg, Dray and Robinson, 1962b).

Low density β-lipoproteins form a class of plasma proteins of mo-

TABLE 1—*Family Studies of Ag (a) Phenotype (Blumberg and Allison, 1961)*

Mating Type	No. of Families		Ag(a+) Offspr.		Ag(a−) Offspr.	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
U. S. White						
Ag(a+) x Ag(a+)	9	7.6	15	14.3	2	2.7
Ag(a+) x Ag(a−)	10	12.4	9	13.2	13	8.8
Ag(a−) x Ag(a−)	6	5.0	0	0.0	13	13.0
Total	25	25.0	24	27.5	28	24.5
U. S. Negro						
Ag(a+) x Ag(a+)	9	8.3	29	29.4	6	5.6
Ag(a+) x Ag(a−)	8	11.8	20	19.2	12	12.8
Ag(a−) x Ag(a−)	7	9.9	0	0.0	18	18.0
Total	24	24.0	49	48.6	36	36.4
Micronesian						
Ag(a+) x Ag(a+)	9	8.6	24	22.5	1	1.5

The number expected is calculated from the Ag (a+) phenotype frequency in the population and the hypothesis that such subjects are homozygous or heterozygous for a gene Ag^A.

lecular weight over one million and containing about 75 per cent lipid (Lindgren and Nichols, 1960). All lipoproteins contain significant amounts of carbohydrate; 3.2 per cent of hexose is associated with the protein in the case of β-lipoproteins (Winzler, 1960). Since many glycolipids are highly antigenic, it is possible that the carbohydrate component contributes to the antigenicity of the lipoprotein.

Inheritance of the Antigen

Subjects reacting with the C. de B. antiserum were classified as Ag (a+), those giving no reactions as Ag (a−). All of the offspring of Ag (a−) x Ag (a−) matings were Ag (a−) (Allison and Blumberg, 1961; Blumberg and Allison, 1961; Blumberg, Bernanke and Allison, 1962a). This suggested that the gene determining the antigen follows mendelian segregation, with negative subjects homozygous for a recessive gene *Ag* and positive subjects homozygous or heterozygous for an allelic gene Ag^A. Family data on U. S. whites and Negroes (Table 1) were consistent with this interpretation. Comparisons of observed and expected numbers of Ag (a−) offspring made by the method of Smith (1956), presented in Table 2, showed satisfactory agreement between the two. Of 27 nonidentical twin pairs tested, 17 had the same Ag

TABLE 2—*Observed and Expected Number of Ag (a−) Children in a U. S. Negro Population, Calculated by Methods of Smith (1956) (From Blumberg, Bernanke and Allison, 1962a)*

Test Comparison	Mating	χ^2	d.f.
Observed no. of Ag (a−) children, given no. of families with Ag (a−) children	Ag (a+) x Ag (a−)	.174	1
Observed no. of Ag (a−) children, given no. of families with Ag (a−)	Ag (a+) x Ag (a+)	.870	1
Observed no. of families with at least one Ag (a−) child, given total no. of families	Ag (a+) x Ag (a−)	.006	1
Observed no. of families with at least one Ag (a−) child, given total no. of families	Ag (a+) x Ag (a+)	.099	1
Total	$\chi^2 = 1.149$ $0.9 > p > 0.8$	d.f. = 4	

type and 10 did not. Of 18 supposedly identical twin pairs 17 pairs had the same Ag type, the discordant twin pair having possibly been nonidentical (from blood group data and haptoglobin typing there was a probability of about 0.3 that this was so). The highly significant difference in concordance rate between identical and nonidentical twin pairs provided further evidence that the antigens are inherited.

Other Antisera

Blumberg, Bernanke and Allison (1962a) reported the finding among 109 sera from transfused subjects of five with allotypic antibodies against β-lipoprotein. Blumberg and co-workers (1964) described the finding of a total of 17 such antisera. Other antisera in transfused subjects have been described by Moor-Jankowski (1962), Bundschuh et al. (1963), Hirschfeld and Blombäck (1964), Berg et al. (private communications).

Further studies were reported on a second antiserum (N. Y.) (Blumberg and Riddell, 1963). Again the antigen reacting with the serum was shown to be a low density β-lipoprotein. A higher proportion of American Negro and American white subjects reacted with the N. Y. antiserum (99.3 and 96.7 per cent respectively) than with the C. de B. antiserum (67.8 and 59.2 per cent, see Table 8). Reactors were described as Ag (b+), nonreactors as Ag (b−). Family studies were consistent with the interpretation that individuals homozygous or heterozygous for a gene Ag^B are Ag (b+). All offspring of Ag (b−) x Ag (b−) matings were Ag (b−). When antisera C. de B. and N.Y. were allowed to react

with each other, two precipitation lines were obtained, showing that the former was Ag (a−b+) while the latter was Ag (a+b−).

Further studies with 11 antisera were subsequently reported (Blumberg et al., 1964). Three techniques were used: cross precipitation, mutual absorptions and population studies. If two antisera gave precipitation with one another, they were classified as different. If they did not react, they were compared by the mutual absorption experiments. If this still did not give evidence of any difference, the population studies were utilized. If this failed to show any distinction, the two antisera were tentatively classified under the same heading. From the results it was concluded that the 11 sera tested had at least five and possibly seven different specificities.

Since there are several, perhaps many, specificities on the serum lipoproteins it was clear that more than one specificity could develop in patients receiving multiple transfusions. By Ouchterlony and absorption experiments it was suggested that more than one specificity existed in the C. de B. (Blumberg, 1963a) and New York antisera (Blumberg et al., 1964a). Allison and Blumberg (1961) had originally reported two types of reactions, strong and weak, with the C. de B. antiserum. When the precipitates were stained with Sudan Black, a reaction of partial identity was noted between the precipitation lines produced with some positive subjects, and a reaction of nonidentity between others (Blumberg, 1963a). Reactions of partial identity were also seen in independent observations using a slightly different technique (Hirschfeld, 1963).

More detailed studies were published by Hirschfeld et al. (1964) who presented an analysis of the reactions with the C. de B. antiserum and the antiserum L.L. found by Hirschfeld and Blombäck (1964). The antilipoprotein allotypic serum L.L. was found to react with some, but not all sera belonging to type Ag (a+) (Hirschfeld, 1963; Hirschfeld and Blombäck, 1964). In most (if not all) cases sera giving precipitates with L.L. belonged to the strongly reacting Ag (a+) type. So far, no Ag (a−) sera have been found to react with serum L.L. among sera from 152 unrelated Swedish individuals tested with both serum C. de B. and L.L. (Table 3). The simplest interpretation of the findings is that: (a) 'strong' and 'weak' Ag (a+) sera have different antigenic determinants; (b) antiserum C. de B. has antibodies against both these determinants; and (c) antiserum L.L. has antibodies against only one of these determinants. To test this interpretation, serum C. de B. was absorbed with a 'weak' Ag (a+) serum using the intrabasin-gel ab-

TABLE 3—*Reaction Patterns of 152 Sera from Unrelated Individuals Against Anti-Ag Sera C. de B. and L.L. (Hirschfeld et al., 1964)*

C. de B.	L.L.	No. of Sera Found
+	+	60
+	−	46
−	−	46
−	+	0
		152

TABLE 4—*Preliminary Designation for the Different Phenotypes Revealed by Ag-Testing with Serum C. de B. (unabsorbed) and Serum L.L. (Hirschfeld et al., 1964)*

C. de B.	L.L.	Denotation
+	+	$Ag(a_1+x+)$ or (a_1-x+)
+	−	$Ag(a_1+x-)$
−	−	$Ag(a_1-x-)$
−	+	Not observed

sorption technique. The serum C. de B. so absorbed now reacted only with 'strong' Ag (a+) sera in a manner identical with serum L.L. Denoting the two antigenic determinates as a_1 and x, and assuming that C. de B. has anti-a_1 and anti-x, whereas L.L. has only anti-x, the preliminary designation for the different reaction patterns is given in Table 4.

During investigation of 152 sera from unrelated individuals by the technique of Hirschfeld (1963) two sera were found to give reactions of partial identity with two other sera when tested against serum C. de B. All four sera were negative against serum L.L. Absorption of serum C. de B. with one of these sera (assumed to lack one antigenic determinant present in the other) showed that C. de B. has, in addition, a third kind of antibody directed against a third antigenic determinant. Using the preliminary notations a_1, x and z for the three antigenic determinants so far demonstrated by serum C. de B., the designations for different reaction patterns observed against C. de B., absorbed C. de B. and serum L.L. are summarized in Table 5.

Full Ag typing of sera from 92 unrelated individuals in the Stockholm area has been carried out by Hirschfeld (Table 6), who has found that if consideration is taken only of phenotypes x and z (which can be unambiguously identified) the proportions observed agree satisfactorily with expectation from segregation of three alleles, Ag^x, Ag^z and Ag^o (the latter manifesting itself as absence of antigens x and z). When further data are available, more exact calculations by maximum likelihood methods can be made.

TABLE 5—*Possible Denotations for the Serum Types Revealed by Antiserum C. de B. Unabsorbed, Absorbed and Antiserum L.L. Unabsorbed (Hirschfeld et al., 1964)*

(anti-a_1) (anti-x) (anti-z)	Absorbed C. de B. (anti-x) (anti-z)	L.L. (anti-x)	Notation
+	−	−	$Ag(a_1+x-z-)$
+	+	−	$Ag(a_1+x-z+)$ $Ag(a_1-x-z+)$
−	−	−	$Ag(a_1-x-z-)$
+	+	+	$Ag(a_1+x+z+)$ $Ag(a_1+x+z-)$ $Ag(a_1-x+z+)$ $Ag(a_1-x+z-)$

Presumed antibodies in these three reagents are shown within parentheses. Only reaction patterns which have actually been observed are given.

Because the $a_1 - x + z +$ phenotype has not yet been observed, it has not been possible to absorb antiserum C. de B. so as to obtain a specific anti-a_1 serum. However, it is already clear that the $a_1 + x + z +$ phenotype exists, so that the three antigens a_1, x and z cannot be due to the presence of allelic genes, only two of which could coexist in any individual. Nevertheless, the a_1 antigen does not appear to be independent of the others, since it is found much more commonly in x + than in x − subjects. Further work will be necessary to elucidate these relationships, although possible analogies are known in other systems, e.g. the presence of the H (O) blood-group antigen in AB subjects who lack the O gene (Race and Sanger, 1960).

It is thus quite clear that the original C. de B. serum has antibodies against several determinants and the same is probably true of other sera. As Hirschfeld (1963) points out, various combinations of three antisera (anti-a_1, anti-x and anti-z) can give rise to seven apparently different antiserum specificities (+++), ++−), (+−+), (−++), (+−−), (−+−) and (−−+). It is theoretically possible that among seven antisera where each specificity is represented, each one will form a precipitate with each other of the other six. The five or possibly seven different specificities reported by Blumberg et al. (1964) might thus be explained by the presence of three determinants, although the actual position may be more complex. It should be recalled that in other systems, for instance with human leukocyte isoantigens, it is the rule for antisera to show reactions against more than one antigenic determinant, and the combined use of population studies, absorption tests and

TABLE 6—*Ag Phenotypes Observed in 92 Sera from Stockholm using Antiserum L.L. for Determining Antigen x and Antiserum C. de B. with and without Absorption for Determination of Other Antigens (Hirschfeld, unpublished). Frequencies of x and z Expected on a Three Allele Theory Have Been Calculated from Uncorrected Formulae of Bernstein (1930) (Two methods of calculation have been employed)*

Ag Phenotype	% Frequency	x and z Phenotypes	% Observed	% Expected (1)	% Expected (2)
$a_1 + x + z +$ or $a_1 - x + z +$	3.97	$x + z +$	3.97	5.83	6.90
$a_1 + x + z -$	33.88	$x + z -$	35.76	33.25	35.75
$a_1 - x + z -$	1.88				
$a_1 + x - z +$ or $a_1 - x - z +$	20.41	$x - z +$	20.41	18.17	20.40
$a_1 + x - z -$	8.16	$x - z -$	39.86	39.87	39.87
$a_1 - x - z -$	31.70				
Total	100.0		100.00	97.12	102.92

Method (1)

Gene frequency $0 = \sqrt{\overline{x - z -}} = 0.6314$

Gene frequency $x = 1 - \sqrt{\overline{x - z -} + \overline{x - z +}} = 0.2237$

Gene frequency $z = 1 - \sqrt{\overline{x - z -} + \overline{x + z -}} = 0.1304$

0.9855

Method (2)

Gene frequency $0 = \sqrt{\overline{x - z -}} = 0.6314$

Gene frequency $x = \sqrt{\overline{x + z -} + \overline{x - z -}} - \sqrt{\overline{x - z -}} = 0.2382$

Gene frequency $z = \sqrt{\overline{x - z +} + \overline{x - z -}} - \sqrt{\overline{x - z -}} = 0.1449$

1.0145

theoretical analyses with an electronic computer has been necessary to sort out their relationships (Payne et al., 1964). Similar studies with the antilipoprotein allotypic sera must be undertaken before any definite conclusions as to allelism or independence of the genes concerned can be made.

Some other relationships are beginning to emerge. Blumberg et al. (1964) found that 3 of the 10 human antilipoprotein antisera which they studied could not be shown to have a specificity different from that in C. de B. Preliminary tests on panels of sera by Hirschfeld and by Allison suggest that Bundschuh's antisera E.Z. and P.O. have the same specificity as antiserum L.L. (anti-x). The reactions with P.O. were rather weak, which might explain why a small proportion of L.L.-positive bloods were negative when tested with P.O.; so far (in about 100 subjects) no individuals who were C. de B.- or L.L.-negative gave positive reactions with E.Z. or P.O. Hirschfeld (unpublished) has found three other sera from Swedish hemophilics giving lipoprotein precipitation with the same specificity as serum L.L. It would thus appear that, at least in northwest Europeans, antibodies against the lipoprotein antigen Ag (x) are relatively often formed. It will be obvious that the subjects originally classified as Ag (a−) will be homozygous for the absence of at least three genetic factors (determining specificities a_1, x and z).

Differentiation of Human Beta-Lipoprotein Types with Animal Antisera

Because serum from multiply transfused subjects is in short supply, it would clearly be valuable to have specific animal antisera for typing. Blumberg, Dray and Robinson (1962b) immunized two chimpanzees with human serum of type Ag (a−) with Freund's adjuvant, but the antibodies obtained reacted with all human sera tested. Moor-Jankowski (1962) and Moor-Jankowski and Brown (1963) immunized *Papio cynocephalus* with the whole serum from an individual human donor and produced an antiserum which reacted differently with different human sera. Berg (1963) immunized rabbits by intravenous injection of isolated human serum beta-lipoprotein from a single individual. The antisera obtained, when absorbed with individual human sera, still reacted wth the lipoproteins from some human subjects, but not with others. Family studies (Berg, 1963; Berg and Mohr, 1963) showed that the specific antigenic determinant defined in this way, which was called the Lp (a) factor, was inherited, all offspring of Lp (a−) x Lp (a−) matings being Lp (a−). Of 314 human sera tested 107 (34.1 per cent) were Lp (a+).

Twenty three sera typed for Lp were tested by Blumberg with antiserum C. de B., and no apparent relationship was found. Berg tentatively concluded that another genetic locus, *Lp*, might be involved. No linkage between the *Lp* locus and Hp or Gc types or blood factors A, A_1, B, M, N, S, C, c, D, E, e, Le (a), Lu (a), P, Fy (a), K, Jk (a), Gm (r), Gm (x) and Gm (b) were observed by Mohr and Berg (1963).

This discussion raises the problem of terminology. Blumberg et al. (1964) have suggested that since it is now known that lipoproteins are involved, the genetic loci controlling the polymorphism might be called *Lpa*, *Lpb*, *Lpc* etc., with superscript numbers for the alleles i.e. Lpa^1, Lpa^2, etc. Further work during the next few years should clarify the genetic situation, and then a meeting of all interested parties could be held to agree on a final notation.

Factors Affecting Lipoprotein Isoimmunization

The first antibody was discovered in a patient (C. de B.) with a refractory hypoplastic anemia of unknown etiology who had received about 50 blood transfusions (Allison and Blumberg, 1961). Blumberg, Bernanke and Allison (1962a) reported the finding of five antisera among 109 sera from transfused subjects. No antibodies were found in tests of sera from 163 subjects who had not received transfusions, and this significant difference from the other series ($p < 0.01$) strongly suggested that the antibodies had indeed appeared as a result of the transfusions. Transfusion introduces a foreign isoantigen, and the production of isoantibodies is similar to that found in laboratory animals given serum from other animals of the same species with or without adjuvants. The low density β-lipoprotein is well known to be one of the most antigenic of serum proteins when whole serum from one species is injected into that of another, and isoimmunization against low density β-lipoproteins has been achieved in rabbits and monkeys (see below). These animal experiments are closely analogous to what happens in man.

Blumberg et al. (1964a) have summarized evidence that the number of transfusions plays an important role in the formation of antibodies, at least in the case of patients with thalassemia (Table 7). Antibodies were never detected in patients who had received less than approximately 20 transfusions. Two patients who did not have antibodies when first investigated developed them after subsequent transfusions. There was no significant correlation between sex or age and the development of antibodies. The panel of 47 subjects with thalassemia major showed a significantly higher incidence of antibodies (29.8 per cent) than in the nonthalassemia group (3.8 per cent). When only those receiving

TABLE 7—*Comparison of Thalassemia and Nonthalassemia Patients in Respect of Frequency of Antibody Formation (Blumberg et al., 1964a) (Anti-LP = Antilipoprotein Precipitin)*

Thalassemia					Nonthalassemia				
Number of Transfusions		Anti-LP			Number of Transfusions		Anti-LP		
Range	Median	Pos.	Neg.	%+	Range	Median	Pos.	Neg.	%+
5–50	20.0	1	10	9	5–50	10.0	1	67	1
51–100	65.5	2	8	20	51–100	65.0	2	11	15
101–200	146.5	5	9	36	101–200	177.5	1	7	13
<200	269.5	6	6	50	<200	300.0	0	13	0
Total		14	33	29.8	Total		4	98	3.9

more than 50 transfusions were considered, there was still a higher proportion of antibodies in the thalassemic patients (13 out of 36) than in nonthalassemic subjects (3 out of 34). The reason for the high frequency of antibodies in the thalassemia patients is still unknown.

In the thalassemic group studied by Blumberg et al. (1964a) the frequency of antibodies formed against lipoproteins (29.8 per cent) was of the same order as the frequency of antileukocyte (37.0 per cent) and antiplatelet antibodies (32.6 per cent), but higher than that of antierythrocyte antibodies (12.8 per cent). The number of anticellular antibodies found in the thalassemic patients was not greatly different from that reported by André, Dreyfus and Salmon (1958) and Dausset, Colin and Colombani (1960) for other transfused patients when arranged according to the number of transfusions. No evidence was obtained that patients who developed lipoprotein antibodies were more apt to develop antibodies to blood cells. Hence, it seems unlikely that thalassemia patients are better antibody formers than patients with other diseases independent of the antigenic stimulus.

No evidence was obtained that anticellular and antilipoprotein antibodies were related. Twenty sera with antileukocyte or antiplatelet antibodies showed no lipoprotein precipitation. One serum had antilipoprotein, antileukocyte and antiplatelet activities. Complete absorption of the anticellular antibodies had no effect on the capacity of the serum to precipitate lipoprotein (Blumberg et al., 1964a).

The question arises whether some of the post-transfusion reactions observed in patients without erythrocyte incompatibility could be due to lipoprotein antibodies. Although this may be so in some cases, Blumberg, Bernanke and Allison (1962a) reported that mild pyrexic reactions were seen in patient C. de B. after he received Ag (a−) as well as after Ag (a+) blood.

TABLE 8—*Frequencies of Subjects in Various Populations Reacting with Different Antilipoprotein Sera*

Serum	C. de B.			New York			J.B.			A. Di B.			S.L.		
Population	No.	%+	Ref.	No.	%+	Ref.	No.	%+	Ref.	No.	%+	Ref.	No.	%+	Ref.
U. S. White (general)	188	54.0	1												
U. S. White (Md.)	120	59.2	2	116	97.6	2	168	98	3	144	77	3	157	97	3
Greek	203	71.9	2	203	93.1	2									
U. S. Negro, Ga.	149	67.8	2	148	99.3	2									
Eskimo, Alaska	97	81.0	1												
Athabaskan Indian, Alaska	102	68.0	1												
Naskapi-Montagis Indian, Labrador	234	97.0	3	103	85.0	3	234	93	3	91	73	3	93	95	3
Quechua Indian, Peru	102	69.6	2	88	86.3	2									
Sioux Indian, U. S.	143	90.9	2	111	77.6	2									
Micronesian, Rongelap	189	98.4	2	82	43.4	2	54	98	3	45	24	3	50	24	3
Polynesian, Bora Bora	96	100.0	1												
Vietnamese	99	71.0	1												

References. (1) Blumberg, Bernanke and Allison (1962a); (2) Blumberg and Riddell (1963); (3) Blumberg (1964).

Nearly all patients with antilipoprotein antibodies reported by Blumberg et al. (1964) continued to receive transfusions. There was no evidence that they had acute, severe, transfusion reactions more frequently than did the patients who did not develop detectable antibodies. Hence, there does not seem to be a good correlation between formation of precipitating lipoprotein antibodies and acute severe post transfusion reactions. There are however, indications that there may be some chronic adverse effect of continued transfusions in the patients who develop antilipoprotein antibodies as well as anticell antibodies. There appears to be an increased morbidity in the thalassemia patients who develop antibodies as compared to those who do not (Blumberg, in preparation).

Frequencies of Lipoprotein Antigens in Different Populations

Population studies are summarized in Table 8. Considerable variations in reaction rates with samples from different human populations were found with almost all sera. Reaction rates were often high, which supports the conclusion that many of the sera probably contain two or more antibody specificities, and further studies with absorbed sera are necessary before the frequencies of individual antigens in different populations can be determined.

From reactions with antiserum L.L., the frequency of Ag (x+) subjects in the Swedish population is 38.9 per cent and the frequency of the gene Ag^x is $1 - \sqrt{0.611} = 0.218$ (Hirschfeld, 1963). Of 72 sera from Bangkok, Thailand, tested by Hirschfeld and Singhrasert (private communication) 65 were positive, giving an Ag (a+) frequency of 90.3 per cent and a gene frequency $Ag^x = 0.689$. If these results are representative, considerable variations in Ag^x frequency in different human populations can be expected.

Mother-Child Correlations

Sera from 81 pairs of mothers and newborn or young infants were examined with antiserum C. de B. (Blumberg, Bernanke and Allison 1962a). A large deficiency of the combination Ag (a−) mother, Ag (a+) infant was found as compared with the expectation from random segregation with full expression of genes at birth. Sera from infants were studied at different ages. One serum which had been negative at birth was positive at 57 days, and six others which had been negative at birth were positive at 2 years (no samples were available from intermediate periods). It seems that the β-lipoprotein carrying Ag specificity, like haptoglobins, gamma globulins and some other serum proteins, is

quite often absent at birth (or not present in detectable amounts) and appears in the blood of infants some time after birth. Many of the offspring of Ag (a+) mothers were Ag (a+) at birth, which suggested that in some cases, the specific β-lipoprotein crossed the placenta from mother to fetus.

Further studies of sera of mothers and offspring with the N.Y. antiserum were reported by Blumberg and Riddell (1963). Similar deviations from random expectation were found—none of the negative mothers had positive offspring, and far too many of the positive mothers had negative offspring. On retesting later (between 49 days and 2 years) 7 nonreacting children (offspring of Ag [a+] mothers) became reactors, showing that the appearance of the specific lipoprotein in the circulation had been delayed until some time after birth.

Lipoprotein Antigens in Transudates

Blumberg, Bernanke and Allison (1962a) reported the finding of material reacting with antiserum C. de B. in synovial fluid from the knees of two subjects with gout; in one there was no evidence of involvement of the knee, so the specific lipoprotein may be a normal constituent of synovial fluid. On the other hand, no Ag (a+) lipoprotein could be detected in cerebrospinal fluid of six Ag (a+) subjects even after concentration 100 times.

Enzymatic Activity Associated with Lipoprotein

Uriel (1961) demonstrated that human serum β-lipoproteins have esterase activity. Lawrence and Melnick (1961) have shown that β-lipoproteins have several other enzymatic activities, and suggest that this may be due to the capacity of lipoprotein to bind enzymes. Precipitates formed by the C. de B. and N.Y. antisera show β-naphthyl acetate esterase activity (Blumberg and Riddell, 1963; Blumberg, 1963).

Lipoprotein Types in Relation to Disease

In view of the fact that polymorphisms are probably maintained in populations by selection (Allison, 1964; Blumberg, 1964), the possible relationship of lipoprotein types to disease has been investigated, special attention being given to conditions in which lipoproteins or lipids might be involved. Blumberg, Bernanke and Allison (1962a) found in relatively small samples no significant differences in age and sex distribution of reactors with antiserum C. de B., and Blumberg, Dray and Robinson (1962b) found no differences in total cholesterol, total phospholipid

or cholesterol/phospholipid ratio in whole serum, low- and high-density fractions of 45 Ag (a+) and 35 Ag (a−) subjects.

Other investigations reported by Blumberg, Ledbetter and Visnich (1964b) showed no differences between proportions of controls reacting with three antisera and groups of patients with coronary artery disease or rheumatic fever. However, there was a significantly higher number of reactors with antiserum C. de B. in a group of American Negro diabetics as compared to an appropriate control group. Similar studies in a Norwegian and Brazilian population did not show such a difference (Berg, Blumberg and Leon, in preparation, 1964).

Congenital absence of β-lipoprotein is associated with acanthocytosis, a hemolytic anemia with abnormal red cells (Salt et al., 1960). A precipitin-like material was detected in the sera of two patients with this disease (Blumberg, 1963b) but this appears to be a nonspecific reaction (Murray and Blumberg, 1965, in preparation).

Allotypes of β-Lipoprotein in Experimental Animals

Dray and Young (1958) immunized rabbits with serum from other rabbits and obtained in addition to γ-globulin antibodies precipitins that reacted with other components of the serum of donor animals. One of the antigenic components had the electrophoretic mobility and appearance of β-lipoprotein (Fig. 4 of Dray and Young, 1958). Dray and coworkers (1962, unpublished) showed by ultracentrifugation and lipid staining that the antigen reacting with the allotypic antiserum was a low-density β-lipoprotein. Moor-Jankowski (1962) and Greuter and Bütler (1963) reported the formation of isoantibodies in primates immunized with homologous serum. One of the isoantibodies in a rhesus monkey studied by Greuter and Bütler was shown to be directed against low density β-lipoprotein.

Isoimmunization Involving Other Human Plasma Proteins

Allen and Kunkel (1963a,b) reported that 35 out of 52 sera from children who had received multiple transfusions contained antibodies against a Gm factor absent from their own γ-globulin. Each of these antibodies was highly specific for a single Gm factor, and all proved useful as reagents for genetic typing. The majority of antibodies were anti-Gm (a), the incidence among Gm (a−) subjects being 85 per cent. Anti-Gm (b) and anti-Gm (x) antibodies were also found. Agglutinating titers up to 1/2,560 were encountered, but no precipitating antibodies. Such antibodies were not found in sera from nontransfused

subjects, and the accumulated evidence indicated that they had been induced by the foreign gamma globulin isoantigens received at transfusion. Tests of antibodies for mercaptoethanol sensitivity and density-gradient ultracentrifugation showed that the great majority were γM (19 S)—unlike the antilipoproteins which are γG (7 S).

One of the anti-Gm sera was in the 7 S class. In addition a different type of antibody was encountered in some of the sera. This failed to react with specific types of γ-globulin, but reacted with antigen-antibody complexes and aggregated γ-globulins. It appeared likely that this group of antibodies arose as a response to antigen-antibody complexes formed through multiple transfusions, whereas the first group arose directly in response to the foreign genetic types of γ-globulin. Steinberg and Wilson (1963) have reported that children may be immunized by Gm factors in their mothers' gamma globulin which crosses the placenta.

The relatively high rate of formation of non-precipitating antibodies against γ-globulins in transfused subjects raises the question whether nonprecipitating antibodies against β-lipoproteins or other plasma constituents may not also be formed more commonly than the precipitating antibodies so far detected. Where antibodies distinguish only a very small number of antigenic determinants they are sometimes nonprecipitating, as in the case of antibodies against insulin (Berson and Yalow, 1962) and those of rabbits recovering from tolerance to heterologous serum proteins (Humphrey, 1964). However, preliminary attempts to find nonprecipitating antibodies in transfused subjects by agglutination of tanned erythrocytes coated with purified β-lipoprotein have so far been unsuccessful (see below).

Isoprecipitins against a serum protein different from low density β-lipoprotein have been found in the sera of some transfused patients, particularly those with hemophilia. The precipitin does not react with any of the sera from normal U. S. populations which have been tested to date. Reactors have been found in frequencies up to 13 per cent in some foreign populations such as Australian Aborigines, Greeks and Taiwanese. The protein has not yet been fully identified and has been tentatively referred to as the "Australia antigen." There is some evidence that the trait is familial. A surprising finding was the very high frequency in patients with leukemia (11.4 per cent of 70 patients) and Down's syndrome (Blumberg and Alter, 1965) whereas it was found in only two other patients (Greeks with thalassemia) out of 659 studied (Blumberg, 1964; Blumberg, Alter and Visnich, 1965; Alter and Blumberg, 1965).

Techniques for Demonstrating Allotypic Antibodies

Sera should be collected before breakfast or as long as possible after the last meal, so as to be free from lipid which can give rise to non-specific, diffuse precipitation in agar gels. The standard test procedure is the Ouchterlony immunodiffusion system in agar. Microtechniques which give very satisfactory results and are economical in serum have been described by Blumberg and Riddell (1963) and Hirschfeld (1963). In the former, clean lantern slides (3¼ x 4″) are covered with a thin layer of 0.2 per cent (w/v) agar in distilled water and dried in an oven at 56°C; this step prevents leakage from the bases of wells which occasionally confuses results. Ten ml. of a 0.9 per cent agar solution in 0.07 M phosphate buffer, pH 7.4, is poured onto the plate and allowed to set, and with a cutter six wells 2 mm. in diameter are placed around a center well of the same diameter. The circumference of the center well is 3 mm. from the circumference of the peripheral wells. A cutter can be made by inserting stainless steel metal tubes or filed off hypodermic needles in a lucite holder. Twenty such patterns, allowing 120 sera to be tested at one time, can be placed on a single lantern slide (Fig. 4). The sera to be tested for antigen are placed in the peripheral wells and 30 minutes later the antiserum is placed in the center wells. The slides are maintained in a humid atmosphere at room temperature, and are viewed by oblique illumination against a matt black background at 16, 24 and 48 hours. Plates are washed in 200 ml. of normal saline (with a few crystals of sodium azide to inhibit bacterial growth) for 2 days with four changes of saline to remove unprecipitated protein. An alternative procedure which shortens the time involved and the risk of dissolving weak precipitates is to soak the agar four times in saline for ½ hour, drying it with squares of Whatman No. 1 filter paper. The slides are fixed in 2 per cent acetic acid (v/v) for 30 minutes, washed in distilled water for 2 hours, covered with a filter paper and dried at room temperature for 24 to 36 hours. The preparations must be completely dry before staining in saturated Sudan Black B (National Aniline) in 60 per cent ethanol for 2 hours. To make the saturated dye solution 1 Gm. of dye is added to 1 L. of 60 per cent ethanol, incubated with occasional stirring at 37° C for 24 hours, cooled to room temperature and filtered. The solution can be kept in dark bottles at room temperature for several months. After staining, slides are washed in two successive baths of 50 per cent ethanol and dried in an oven at 37° C. If desired, they can be counterstained with the general protein stain Azocarmine as described by Uriel (1964), who also gives a convenient

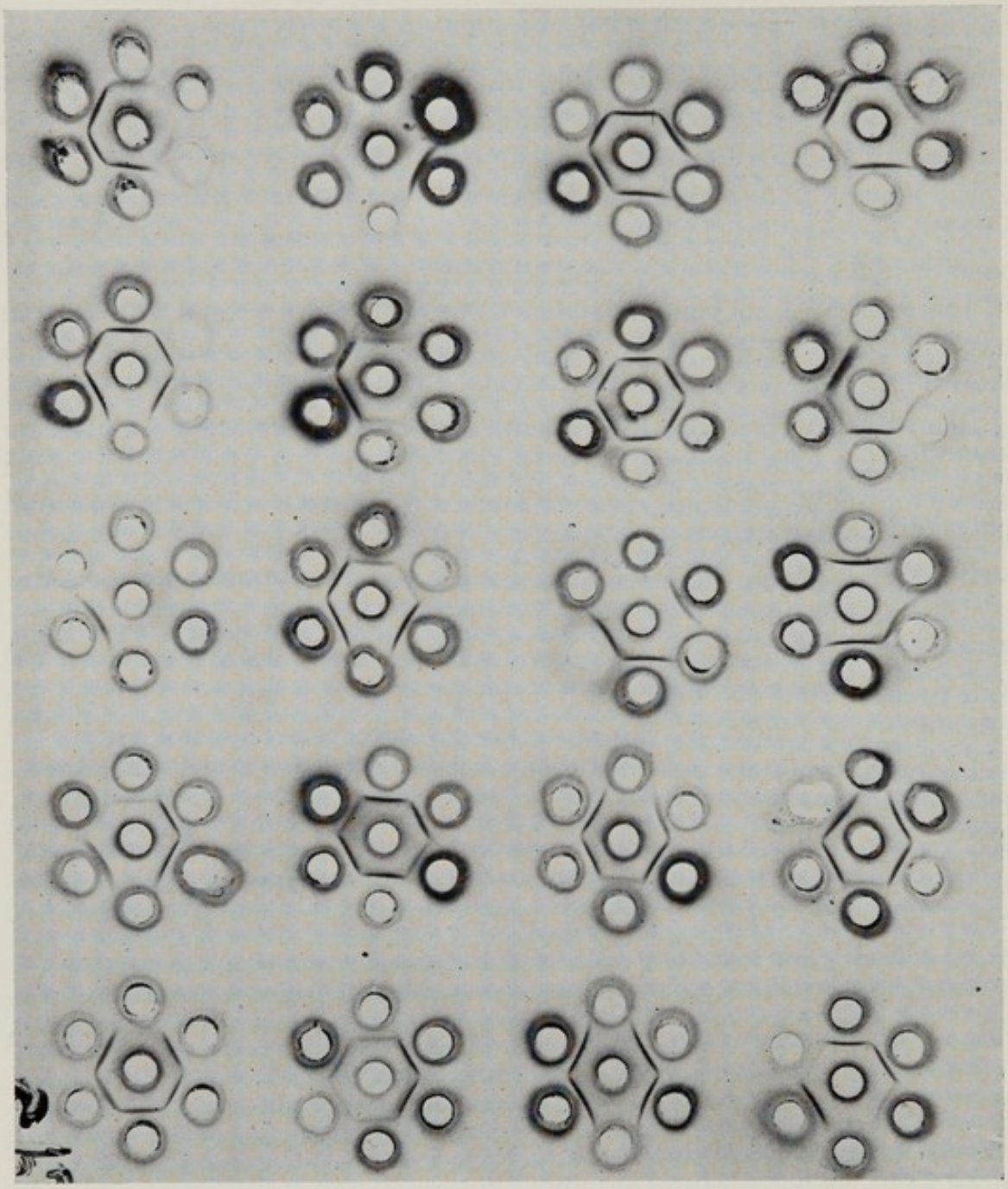

FIG. 4—Micro-Ouchterlony method on 3¼" x 4" lantern slide. The center cup of each 7-hole pattern contains antiserum C. de B. and the peripheral wells contain normal sera the phenotypes of which are to be determined. Using this pattern, 120 sera can be tested in a single operation, and weak and strong reactors can be distinguished, Sudan Black stain (Blumberg et al., 1964a).

method of stripping the dried agar off the glass so that the former can be filed.

Allison and Blumberg (1961) and Hirschfeld (1963) have pointed out that as a rule both the lipoprotein antigens and allotypic antibodies are stable on storage at −20° C. Tests on samples of sera stored as long as 6 years, with repeated thawing, give with good antisera results

identical with those on fresh sera from the same subjects. With weak antisera there appears to be some deterioration on storage of both antigens and antisera. The weak precipitates tend to be dissolved in excess antigen or antibody, so that the precipitation patterns should be observed at intervals from 16 to 48 hours and photographed before staining; otherwise some positive reactions may be missed. Care must be taken to avoid nonspecific lipoprotein precipitation. It is well known that lipoprotein is precipitated by various sulfated polysaccharides (see Cornwell and Kruger, 1961), and many samples of agar contain these compounds. Both Oxoid Ionagar (Oxoid Division, Thames House, Queen's St. Place, London, E.C.4; Consolidated Laboratories, Chicago Heights, Ill.) and Difco Noble-Agar have proved satisfactory for most tests, but with weak antisera a higher proportion of positive reactions has been obtained by using instead of agar 0.7 per cent agarose (L'Industrie Biologique Française, S.A., 35 à 49 Quai Moulin de Cage, Gennevilliers, Seine, France) in phosphate buffer as described by Allison (1965). However, in some cases, precipitin lines seen in Ionagar were not detectable in agarose (Blumberg et al., in preparation).

The possibility of nonspecific precipitation of lipoproteins by bacterial products must also be borne in mind (Teichmann and Vogt, 1964).

It has also been found by Davies et al. (unpublished) that tanned erythrocytes coated with β-lipoprotein of appropriate type are strongly and specifically agglutinated by antiserum C. de B. This technique was designed as a test for nonprecipitating antibodies, but in a limited survey of sera from transfused subjects so far no agglutinating antibodies have been found in sera lacking a precipitin. Allison and Blumberg (1961) found that guinea-pig complement is not fixed when antiserum C. de B. reacts with antigen, nor could a passive cutaneous anaphylactic reaction be obtained in guinea pigs, possibly because the large size of the antigen limits diffusion in the skin.

The techniques for identifying lipoprotein antibodies are straightforward and require very little time, and we hope that they will come into routine use in blood-transfusion laboratories.

Acknowledgment

We are indebted to Dr. J. Hirschfeld for allowing us to quote unpublished observations and for helpful discussion.

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CHAPTER 7

Population Genetic Studies of the Indigenous Peoples of Australia and New Guinea

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DURING THE LAST DECADE fresh impetus has been given to the study of the genetic structure of human populations. The impetus has arisen from the confluence of several streams: the demonstration of the selective significance of abnormal hemoglobins, the development of new techniques for demonstrating serum protein and enzyme polymorphisms, and the desire to provide material to test mathematical models of the effects of inbreeding and of the behavior of genes in human populations. The concomitant increase in research funds and the improvement in means of communication have promoted the growth of interdisciplinary studies and brought relatively distant populations within the reach of laboratories with specialized facilities.

In the last few years, increasing emphasis has been placed on studies of human populations having minimum contact with advanced technologies. A recently published report by a W.H.O. scientific group (W.H.O. 1964) emphasized that the few remaining populations with a simple type of economy represent, both in size and level of technology, the closest approximation one can find to the conditions under which man has lived for the greater part of his existence.

It might appear that the indigenous populations of Australia and New Guinea provide opportunities for detailed studies of the relationship between the genetic structure of relatively isolated human groups and

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that of neighboring groups and of the interactions with disease vectors and other environmental factors. However, social change in both countries is rapidly affecting the indigenous populations. In Australia it may be too late already to unravel many of the factors which have contributed to the observable genetic patterns and in New Guinea the situation will soon be comparable, as the changing economy increases the mobility of individuals and local groups lose their sharp identities. For this reason it is important to review the studies with a genetic orientation which have been undertaken up to the present in these two areas. Future research can then attempt to fill obvious gaps in our knowledge expeditiously.

The General Background

The full-blood Aboriginal population in Australia is estimated at present to be nearly 50,000: this number is unevenly spread over the continent. The greatest density of population is in the belt north of the Tropic of Capricorn, stretching from the Kimberleys of Western Australia across Arnhem Land into Northern Queensland. The southeastern and southwestern areas of the continent have no significant full-blood population remaining and the inhabitants of the central and semi-arid regions are moving out to the peripheries or congregating in relatively permanent settlements associated either with Christian missions or government welfare posts.

The Australian Aboriginal inhabited environments ranging from small areas of tropical rain forest in northeast Queensland to vast areas of arid and semi-arid desert in the interior, from a wide belt of monsoonal summer rain with long dry winters in the north to the well-watered cold winter climates of the south. Many cultural adaptations occurred between these extremes (Berndt and Berndt, 1964) and languages in particular became greatly diversified within the Australian language family. Wurm (1963) estimates that there are some 500 different forms of speech or "communalects" in Australia and that these represent perhaps 150 different languages. Physical differences in stature, head form, hair form and skin color are apparent also. Tindale and Birdsell (1941) claim that the peoples of the Queensland rain forest represent a distinctive group. Birdsell (1949) has argued on the basis of physical differences that the Australian Aborigines are derived from three entirely different parent stocks. A list of publications on the physical anthropology of Australian Aborigines has been given recently by Abbie (1963).

No complete census of the indigenous population of New Guinea has yet been undertaken. Estimates based on official reports suggest

that it approximates three million, two-thirds of which are in the eastern half of the island under Australian administration; the remaining portion is in the western half, presently administered by Indonesia. New Guinea consists of two broad climatic zones; the lowland swampy coastal areas are hot and humid and malaria is holoendemic; the central highlands are more temperate in climate, though rainfall is still high, and malaria is spasmodic or absent. Within these two broad climatic zones, numerous local climatic variations exist, particularly rain shadows created by mountain ridges. These variations require delineation in terms of topography and vegetation before intergroup comparison for some characteristics can be given full significance (Brookfield, 1964).

In many highland areas, dietary problems are acute. Protein is deficient and the shortage of sodium salts produces marked alterations in electrolyte balance (Hipsley and Kirk, 1963). Nearly two-thirds the population live in highland areas.

Even greater contrast in social structure and technology exist in New Guinea than in Australia (Ryan, Healy and Kerr, 1962). Although the different forms of speech have never been completely enumerated, they run into several hundred and they belong to two major language families, Austronesian and non-Austronesian (Capell, 1962). Physical anthropological studies have been limited in scope and almost entirely restricted to populations in the eastern half of the island. In most investigations stature only has been measured, although Hambley (1940) provides extensive data on skulls.

Despite the relative paucity of published information on the anthropometry of populations in Australia and New Guinea, extensive studies of the distribution of genes controlling various blood groups, serum protein groups and enzyme groups have been undertaken. The majority of workers in Australia have summarized their results for these studies on a regional or location basis rather than by tribal or language-group affiliation. This is due partly to the failure by earlier investigators to realize the biological importance of the tribal unit, or the 'horde' within the tribe, and partly to the rapid breakdown of tribal boundaries following European contact. The early work in New Guinea was deficient also in this respect, but the majority of recent studies have had their results tabulated by linguistic affiliation.

In the following sections an attempt will be made to summarize the available information for the blood group, serum group and enzyme group systems, together with the results of investigations on a number of other simply inherited characteristics. In addition a brief summary is included on abnormal hemoglobins, thalassemia and G-6-PD deficiency

and also of the disease, Kuru, which is highly localized in New Guinea and which may have a genetic basis. Anthropometric studies and studies of other morphologic characters such as skin color, hair form, and dermatoglyphs as well as investigations of health, including both biochemical and physiological adaptation to varying environmental situations have been excluded from this review.

Blood Groups

The ABO Blood Groups

The earliest studies of the distribution of the ABO blood groups among the indigenous inhabitants of Australia and New Guinea were carried out more than 40 years ago (Tebbutt and McConnell, 1922; Heydon and Murphy, 1924). Early work has been summarized by Boyd (1939). Simmons et al. (1961, 1964) have brought the summary up-to-date for New Guinea and Australia. Kirk (1965) has provided a complete tabulation of all blood group results for Australia.

Table 1 summarizes the range of gene frequencies for the ABO, MNSs and Rh blood group systems in Australia and New Guinea. For convenience both countries have been divided into arbitrary geographical regions, not all of which coincide with administrative regions. These arbitrary regions, together with localities mentioned in the text, are indicated on Maps 1 and 2. In addition, for New Guinea some results are given for separate linguistic groups.

The frequency ranges are based on the figures for discrete populations listed in all published studies, together with unpublished material acknowledged in the text. A list of source papers is included at the end of the bibliography. Where more than one investigator has sampled populations in the same area, the gene frequencies determined in each case are included in the stated range.

There are marked contrasts between Australia and New Guinea in the distribution of the ABO blood group genes. Only blood groups O and A are present over the greater part of Australia, but the frequencies of the blood group O gene and of the A_1 gene are very variable. The highest values of the A_1 gene are found in the desert areas southwest of Alice Springs and extending into Western Australia as far as Leonora; there are correspondingly low values of the O gene in the same area. Peripheral to these desert areas the value of the A_1 gene falls, reaching values less than 25 per cent for almost the entire coastal area from Broome, in Western Australia, along the northern shores and down the east coast as far as New South Wales. In Cape York, the O gene fre-

TABLE 1—*Range of Gene Frequencies (in %) for the ABO, MNSs and Rh Blood Group Systems in Australia and New Guinea*

(For sources see separate list at end.)

Locality or Population	A_1	B	O	MS	Ms	NS	Ns	R_1	R_2	R_0	R_z
Australia:											
Cape York	4–22	0–14	74–96	0	12–34	0	66–88	66–79	6–8	4–25	1–11
S. E. Queensland	18–25	2–4	71–80	0	21–28	0	72–79	66–67	11–12	13–16	6–8
North Australia[a]	8–23	?	77–92	0	27–34	0	66–73	64–69	7–25	6–29	0–2
Central Australia	22–46	0	54–78	0	13–39	0	61–87	56–57	25–41	1–12	0–7
Western Desert	31–50	0	50–69	0	0–10	0	90–100	51–57	38–44	2–3	2–3
Western Central	35–46	0	54–65	0	22–32	0	68–78	57–62	27–29	0–9	2–13
Kimberleys	11–23	0	77–89	0	22–41	0	59–78	55–76	13–28	4–14	2–9
Southern Gulf of Carpentaria	0–10	0–24	76–93	0	31–51	0	49–69	52–67	5–24	9–43	0–1
New Guinea:											
Gulf of Papua	18–25	11–20	62–67	0–5	4–23	8–10	71–86	95	2–4	1–2	0–2
Port Moresby Area	10–13	16–18	69–72	0	18–24	6–9	70–73	86–94	6–14	0	0–0.1
Milne Bay	20	10	70	0	27	15	57	91	4	4	1
Bulolo Valley	27–32	16–17	51–57	0	10–16	10–12	72–80	87–94	4–13	0–2	0
Sepik River	20–45	4–26	41–66	0–6	3–18	6–17	70–75	81–94	4–15	0–4	0
Sarmi	27	12	61	0	28	0.5	71	95	4	1	0
Nimboran-Sentani-Sekori	19–30	8–10	60–72	0	3–11	0–2	87–97	80–96	4–12	0–8	0
Is. of Schouten	10	12	78	0	27	4	69	93	6	1	0.3
Asmat	28–30	20–22	49–53	0	0–4	2–8	89–98	96–100	0.3–4	0–0.7	0
Djar, Mandobo and Moejoe	7–19	5–12	73–83	0	4–6	10–28	64–85	86–95	3–10	2–9	0
Merauke Area	18	9	73	0	6	10	84	88	8	1	2

TABLE 1—*(Continued)*

Goloila	12	9	79	0	6	6	88	97	3	0.7	0
Kukukuku	14–34	2–13	60–74	0	8–10	4–8	83–86	76–85	7–21	3–7	0
Fore	29	10	61	0	3	5	93	93	4	3	0
Eastern Highlands†	11–32	5–22	50–77	0	1–15	0.4–11	82–97	85–98	4–12	0–8	0
Chimbu	20–24	17–18	59–62	0–0.4	3	12–15	83–85	90	8	2–3	0
Goroka	17–20	9–10	71–73	0–0.5	8–11	6–11	78–86	80–84	10–13	6–7	0
Western Highlands‡	15–37	7–22	56–70	0–6§	1–12	9–28	63–90	87–99	1–13	0–5	0
Telefolmin	20	10	70	0	4	1	95	87	6	7	0
Star Mountains	8	18	74	0	2	0	98	97	2	0.6	0.6
Wissel Lakes (Pygmies)	8	14	78	0	10	0.7	89	85	12	3	0
Dani-Mulia and Dani	17–23	19–38	44–58	0	7–9	0	91–93	90–98	2–10	0–3	0
Enga	25	14	61								

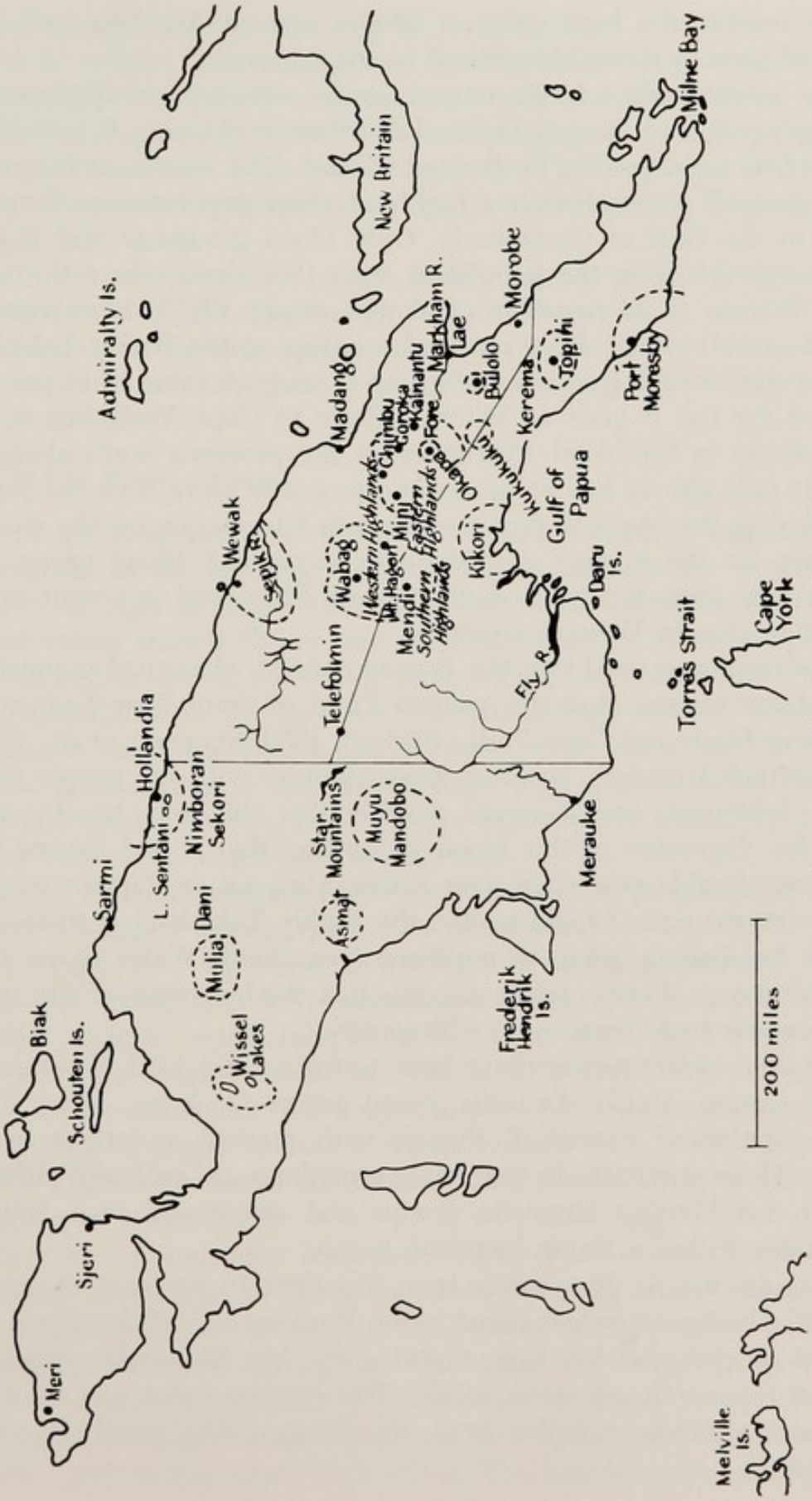
°Including Melville and Bathurst Island.

†Gimi, Keiagana, Kanite, Usurufa, Kamano, Auyana, Agarebi, Awa, Cadsup, Tairora, Yor Pavaian, Oiana.

‡Mt. Hagen, Aiome, Chimbu to Mt. Hagen, Minj.

§0 except among 54 light-skinned natives.

MAP 1—Principal towns and localities in Australia.



MAP 2—Principal towns and localities in New Guinea.

quency reaches the high value of 96 per cent at Aurukun, and high values of gene O prevail in general in coastal areas.

Early investigations in Eastern Australia detected the presence of blood group B, and the geographical distribution of group B individuals was studied subsequently by Birdsell (1950). The maximum frequency of the group B gene occurs in a highly aberrant population on Bentinck Island in the Gulf of Carpentaria. Only blood groups O and B have been detected among the population from this island where the value of the B gene is 24 per cent (Simmons et al., 1962). More recently Simmons et al. (1964) have reported a group on the Barkly Tableland where a similar high frequency of group B exists. A value of 14 per cent is found for the B gene at Mitchell River in Cape York, but it falls progressively in full-blood Aborigines as one proceeds north along the Cape. It falls also to low values as one proceeds away from the Barkly Tableland in the opposite direction, though information on the western boundary for the B gene is inadequate at present. Blood group B is present only sporadically in south Arnhem Land and is absent in the Kimberley area of Western Australia.

It has been suggested that the B gene entered Aboriginal populations from Malay sources through Arnhem Land or from New Guinea via the Torres Strait and Cape York (Birdsell, 1950; Simmons et al., 1958). The Bentinck Islanders, however, have affinities with the people of the Barkly Tableland, and it seems possible that this area has been the center for dispersion of this blood group and that it had existed here for a considerable period of time before Malayan or Papuan contacts of more recent date. Linguistically, the Barkly Tableland is distinctive, but the Bandjalong group in northeast New South Wales shows some similar features. Wurm (1963) suggests that the languages of this group may formerly have been more widespread.

More than 16,000 persons have been tested for the ABO blood groups in New Guinea. Unlike Australia, blood groups O, A and B are found in all populations examined, though with marked variations in frequency. These variations in frequency sometimes are strikingly different between neighboring linguistic groups and sometimes even between populations within a single linguistic group.

Values for the A_1 gene range from less than 10 per cent to over 30 per cent. The lowest values occur in the Western half of the island. The value of 34 per cent has been reported for one Kukukuku population sampled in Lae (Craggs et al., 1958). This extreme value was not found in other Kukukuku samples from the Eastern Highlands (Simmons et al., 1961).

The lowest values for the B gene, ranging from 2 to 7 per cent, are found in several linguistic groups in the Eastern Highlands, and the highest value of 38 per cent occurs among the Dani-Mulia, another highland group in Western New Guinea. In general, however, the values of the B gene range from 5 to 20 per cent. The frequency of the blood group O gene is moderately high and ranges between 50 and 80 per cent in most areas.

With the exception of a single family belonging to the Agarabi linguistic group of the Eastern Highlands (Simmons et al., 1961), the blood group A belongs to the subgroup A_1 throughout New Guinea. Simmons and his colleagues believe that this single family represents a recent mutation.

The MNSs Blood Groups

Both Australia and New Guinea are characterized by extremely high frequencies of the blood group N gene. In Australia the frequency of gene N is high in Cape York, and also in the Western Desert area, where values around 95 per cent are found: for one small sample of 56 persons at Cundeelee, a value of 100 per cent has been found (Vos and Kirk, unpublished). In New Guinea similar high values occur in the Highlands, on Daru Island and in some areas bordering the Gulf of Papua. Immediately to the east of these high gene N areas along the Gulf of Papua, however, is an area of low gene N frequency and low values occur also in the Isles of Schouten.

One of the most striking blood group results is the complete absence of the S antigen among Australian Aborigines. In over 3,000 persons tested, only two S-positive individuals have been detected. One of these was thought to be of mixed parentage and the other was discounted since no repeat sample could be obtained. The s antigen, however, was uniformly present in over 1,400 persons tested from many parts of the continent. Although no tests have been performed with anti-U from a Negro source, it seems unlikely that U-negative persons (S−s−), who are found commonly in African or U. S. Negro populations, occur in Australia.

No S−s− persons have been detected in New Guinea. The S antigen, however, in contrast to Australia, does occur in New Guinea though with variable frequency. Among the Dani-speaking peoples, in the Star Mountains area and among the Nimboran and Sekori peoples, all in West New Guinea, there are populations where the S antigen is absent. On the other hand, in other parts of New Guinea the frequency of S-positive persons may reach high values. The highest value of 50 per cent

occurs at Mandobo in West New Guinea, followed by 41 per cent at Enga, 37 per cent in the Western Highlands and 35 per cent for Chimbu. In general, the highest values for the S antigen are found in the central highland areas with lower values peripherally. But there are marked exceptions, as instanced by the absence of the S-antigen in the Star Mountains. Macintosh et al. (1958) drew attention to the cline from west to east in the S gene frequency in the highland areas. These authors suggest also that the blood group frequencies indicate the possible existence of an earlier population in New Guinea possessing a high S and no M and a later immigrant population with no S and a little M, but an otherwise similar blood group pattern.

The Rh Blood Groups

Four Rh genes, R^1,R^2, R^o and R^z are present in all areas in Australia, but only the first three of these are found in the majority of New Guinea populations. The R^z gene occurs in New Guinea along the western half of the Gulf of Papua, on Daru Island and extending to the vicinity of Merauke. Elsewhere it has been reported from the Star Mountains and the Islands of Schouten. These populations coincide with many of those, as noted above, where the S antigen of the MNSs blood group system is absent. Simmons et al. (1946) believe that the presence of R^z on Daru Island and in neighboring coastal areas is a result of Australian Aboriginal influence. Nijenhuis (1961), however, doubts if the frequency of R^z found in the south coastal areas can be explained solely by race mixing and he suggests that selective factors have been operative also.

The Rh genes show wide fluctuations in frequency in Australia. R^1 ranges from low values around 50 per cent in the Western Desert to the highest value of 80 per cent at Mitchell River in Cape York. The values of R^2 show an inverse relationship in general, ranging from 6 per cent at Mitchell River to the highest value of 44 per cent at Jigalong in Western Australia. R^o has a low value in most of the desert areas, but in contrast R^z achieves its highest values in the same places, and another focus of high R^z values occurs in Cape York.

The frequency of R^o is low or zero in many parts of New Guinea and its highest frequencies, ranging from 5 to 9 per cent, occur in some highland groups. R^z also has low frequency in many parts of New Guinea, its frequency for most populations lying within the range of 5 to 15 per cent. In view, therefore, of the absence of R^z and the low values for R^o and R^2, the predominant Rh gene throughout New Guinea is R^1. In many populations, it has a value around 95 per cent and with few exceptions it always exceeds 85 per cent. The values for R^1 in New

Guinea are the highest in the world and are paralleled only by other Melanesian populations. The highest value for R^1 in Australia just reaches the lower limit for the frequency of this gene in New Guinea.

D^u variants have been reported both from Australia and New Guinea, although in the latter country they appear to be highly localized. A small number has been found among persons belonging to the Gadsup linguistic group in the Eastern Highlands (Simmons et al., 1961; Watson et al., 1961) and Nijenhuis and his colleagues have reported others from the Nimboran and Sentani groups near the north coast of West New Guinea (de Vries and Nijenhuis, 1960; Nijenhuis and de Vries, 1962).

Tests carried out elsewhere in New Guinea for both 'high grade' and 'low grade' D^u variants have failed to reveal their presence. Both types of variants have been reported, however, in Australia (Walsh, 1952; Simmons et al. 1958). The existence of D^u variants in Australia, particularly extreme forms of the 'low grade' type has, for some time, made difficult the assessment of whether the R' gene exists in Australia. Recently Simmons (1958 and personal communication) has reported a a single individual from La Grange in Western Australia, whose cells consistently fail to be agglutinated by any one of a battery of anti-D sera, either by the use of enzyme-treated cells or by use of the indirect antiglobulin test. This person, however, is positive with anti-C and anti-e sera. Vos and Kirk (unpublished) have found a similar person at Sunday Island, in the same geographical region as La Grange. It seems, therefore, that the R' gene does occur in Australia at least in this one area, but its frequency elsewhere must be zero or extremely low.

E^u variants have been reported in West New Guinea by Nijenhuis (1961) and also in Australia (Sanger et al., 1951; Simmons et al., 1958; Vos and Kirk, unpublished). A quite separate variation in the E antigen has been detected in Australia by Vos and Kirk (1962). The specific antibody (anti-E^T) found in an Aborigine from the Western Desert reacts with all E-positive European bloods; in the Western Desert area, however, 35 per cent of E-positive individuals fail to react with the specific antibody. The frequency of negative reactors is lower in other parts of the continent, and in Cape York only 1 per cent of the E-positive individuals failed to react with anti-E^T.

Simmons et al. (1961) has carried out a small number of tests in New Guinea with anti-V and Vos and Kirk (unpublished) have carried out similar tests in the Western Desert of Australia. This antiserum reacts with 28 per cent of U. S. Negroes in cases where R^o or r are present. Despite the presence of R^o in both New Guinea and Australia, the tests with anti-V in both places were uniformly negative.

TABLE 2—*Range of Phenotype Frequencies (in %) for Other Blood Group Systems in Australia and New Guinea*

Locality or Population	Le(a+)	Le(b+)	P_1(+)	Fy(a+)	Lu(a+)	K(+)	Di(a+)	Js(a+)	Jk(a+)	Wr(a+)	Wb(+)
Australia:											
Cape York	8–21	–	29–32	100	–	–	0	–	–	0	–
S. E. Queensland	–	–	–	–	0	–	–	–	–	–	–
North Australia	–	–	–	–	–	–	–	–	–	–	–
Central Australia	0–7	–	18–65	100	–	0	0	–	–	–	–
Western Desert	5	–	44–73	100	–	0	0	0	–	0	–
Western Central	14–21	–	26–28	–	–	–	–	–	–	–	–
Kimberleys	0–19	–	24–61	100	0	0	0	–	–	0	–
Southern Gulf of Carpentaria	0–9	–	9–27	100	–	–	–	–	61–69	–	0
New Guinea:											
Eastern Highlands	0	82	37	100	0	0	0	0	86	–	–
Western Highlands	0	–	57	100	–	1	–	–	–	–	–
Goroka	0	–	57	100	–	2	–	–	–	–	–
Fore	0	90	41	100	–	0	0	0	85	–	–
Enga	0	59	43	100	0	0	0	0	92	–	–
Is. of Schouten	–	–	43	100	0	0.6	–	–	–	–	–
Asmat	–	–	58	100	–	–	–	–	–	–	–
Dani-Mulia	3	–	50	100	0	0	0	–	–	–	–
Nimboran-Sentani-Sekori	–	–	49–66	100	–	0	0	–	–	–	–
Wissel Lakes (Pygmies)	0	–	63	90°	–	–	–	–	–	–	–

°Tested after 6 months storage.

A singularly interesting discovery in the Rh blood group system was reported by Vos et al. (1961). The red cells from a 37 year old Aboriginal woman from the Western Desert failed to react with all known types of Rh antisera. Tests on more than 500 persons from the same population have failed to reveal another example of Rh ---/---. Another case of Rh ---/--- has been reported recently in the United States (Levine et al., 1964).

Other Blood Group Systems

Table 2 summarizes the phenotype frequencies for a number of other blood group systems. In general only a relatively small number of persons has been tested for some of the antigens in each of these systems, but despite this limitation, certain generalizations can be made.

Both in New Guinea and in Australia, 100 per cent of persons tested have reacted positively with anti-Fy^a and have failed to react with anti-K, anti-Lu^a, anti-Di^a and anti-Js^a. Two Di (a+) persons have been reported by Buettner-Janusch et al. (1960) among the Kepauke people in the highlands of West New Guinea. This result is in such marked contrast with all other Diego tests in this area that it merits reinvestigation. Further, in another report Graydon et al. (1958) found only 90 per cent Fy (a+) persons among the Wissel Lakes pygmies. The tests with anti-Fy^a were carried out in this case on cells that had been stored in glucose-citrate solution for a period of 6–9 months and it seems reasonable to disregard the results, which the authors themselves advanced with considerable caution. Recently, Nicholls et al. (1965) have reported five persons, apparently of unmixed origin at Amoorgura, who are Kell positive. Analyses of fresh samples from these persons have confirmed the original results. Further Kell typing in this area is clearly needed.

Reactions with anti-P^1 sera show a wide range of frequency for P-positive persons. In Australia higher values, ranging from 18–73 per cent, prevail in the Central Western Desert regions. Values in the Kimberleys range from 24–61 per cent, but in the coastal areas of Western Australia, the Gulf of Carpentaria region and Cape York, values range from 9–32 per cent. In New Guinea, the variation is smaller, the majority of values falling within the range of 40–60 per cent. It is possible that the areas of low P^1-positive frequency in Australia do represent real genetic differences. But tests with anti-P sera are notoriously difficult (see for instance Neel et al., 1964) and the problem is worth reinvestigation.

Similar difficulties apply to tests with anti-Le^a and anti-Le^b sera. In

New Guinea, with the exception of a small number of Dani-speaking persons in the Mulia region, no Le (a+) reactions have been found. In contrast, although some populations in Australia show low frequencies of reactions with anti-Le^a, others as in Cape York and Central Australia show up to 21 per cent positive reactions. To what extent these variations are due to different antisera in testing laboratories, or to varying lengths of storage of cells during transport, is still not clear. The same strictures apply even more strongly to tests with anti-Le^b sera. Frequencies of Le (b+) persons in New Guinea range from 59 per cent for a small sample of Enga to 90 per cent among the Fore.

A small number only of tests for ability to secrete ABH substances has been carried out in Australia and New Guinea. In the latter area, 99–100 per cent of persons tested were secretors (Semple et al., 1956; Graydon et al., 1958): in Australia 97–98 per cent of Aborigines in Central and Western Australia are secretors (Simmons et al., 1954; Vos and Kirk, unpublished).

The Kidd blood group system has been tested for in only one region in Australia, the southern Gulf of Carpentaria. Approximately 65 per cent of persons were Jk (a+) (Simmons et al., 1964). Somewhat higher values have been reported for some highland groups in New Guinea (Simmons et al., 1961). The Kidd system could well become a valuable marker for populations in these two areas.

Tests have been carried out in a number of regions in Australia for the antigens belonging to the "private" blood group systems, Wright and Webb. No Wr (a+) or Wb (+) persons have been found.

Serum Protein Groups

Haptoglobins

The three main haptoglobin phenotypes, Hp 1-1, Hp 2-1 and Hp 2-2 are present in all Australian and New Guinea populations. In addition, there is a low but variable frequency of ahaptoglobinemia (the Hp O phenotype). Unlike the situation among African Negroes, however, the ahaptoglobinemia is not associated with the presence in the population of the Hp 2-1 (Mod) phenotype. No case of this phenotype has been reported from Australia. Barnicot and Kariks (1960) found a single Hp 2-1 (Mod) person in over 500 samples from New Guinea and Curtain et al. (1964) found only two further cases in nearly 2,000 samples. Two persons belonging to the rare "Johnson" phenotype have been recorded from Papunya in Central Australia (Nicholls et al., 1965) but no case has been described from New Guinea.

Within Australia there are significant variations in the range of frequency for the Hp^1 gene, (Table 3). Low values of 10 to 21 per cent prevail in Cape York and in the Western Desert, with slightly higher values in Central Australia. Higher values occur in the Kimberleys, with values of Hp^2 up to 39 per cent in Central Western Australia. In contrast, the values of Hp^1 for all populations in New Guinea greatly exceed the highest Australian value, ranging from 53 per cent in the Eastern Highlands to 82 per cent along the Markham River Valley. These frequencies are amongst the highest in the world.

New Guinea lowland populations, on the average, have higher Hp^1 frequencies than do highland populations, though there is considerable overlap in frequencies between these two situations. In addition, lowland populations have a higher percentage of persons without detectable haptoglobin (Hp O). With one exception, the highland groups either have zero or small percentages of the Hp O phenotype. Barnicot and Kariks (1960), however, found 23 per cent of Hp O in one highland group (Chimbu). This group should certainly be resampled.

Curtain et al. (1964) in a detailed study, find the Hp^1 frequency in the Eastern Highlands is significantly lower at the 1 per cent level than it is in the Markham or Sepik River Valleys. The Hp O phenotype was completely absent in over 1,000 samples from the highlands, but ranged in frequency from 2 to 13 per cent in over 900 samples from the Markham and Sepik River Valleys.

In addition, Curtain and his colleagues find significantly lower quantitative haptoglobin levels in lowland areas compared with highland areas. They suggest that these lowered haptoglobin levels, reflected also in the increased frequency of the Hp O phenotype, may be due to the removal of haptoglobin during intravascular hemolysis. In the Markham and Sepik River Valleys, malaria may be a major cause of this intravascular hemolysis. Since the haptoglobin level in Hp 2-2 persons is normally lower than in either Hp 1-1 or Hp 2-1 persons, loss of haptoglobin will tend to increase the apparent frequency of the Hp^1 gene. Such a mechanism would explain the difference in Hp^1 frequency between lowland and highland areas.

Another possibility discussed by Curtain et al. (1964) is that there is a selective pressure favouring the Hp^1 gene in lowland areas. They state "since sera of type 1-1 and type 2-1 individuals appear to bind more hemoglobin per 100 ml. than does those of type 2-2, this could place the latter at a disadvantage in the maintenance of the delicate iron balance in the presence of hemolysis, intestinal iron loss due to parasitic infestation and dietary iron deficiency." But although it is

TABLE 3—*Range of Gene Frequencies (%) for Serum Protein Groups by Geographical Region in Australia and New Guinea*

Locality	Hp O	Hp^1	Tf^D	Tf^B	Gc^1	Gc^{Ab}	Gm^a	Gm^{ax}	Gm^{ab}	Inv(a+)
Australia:										
Cape York	0–3	10–21	2–11	6	72–90	2–7	62–85	14–34	0–5	39–61
North Australia	–	–	–	–	87	2	–	–	–	–
Central Australia	0–4	13–29	5–10	0	86–94	1–5	62–70	29–37	0–1	37–47
Western Desert	<1	15	20	0	92–98	0–1	73	27	0	49
Western Central	0–1	2[illegible]–39	10–16	0	91–93	2–3	–	–	–	–
Kimberleys	0–1	22–32	7–15	0	81–90	1	58	25	17	45
New Guinea:										
Sepik River	4	70–76	12–14	0	–	–	–	–	–	–
Markham River	2–13	69–82	11–14	?	–	–	–	–	–	–
Eastern Highlands	0–23	59–72	6–14	<1	–	–	45	8	47	7
Southern Highlands	0	64–70	6–14	0	–	–	–	–	–	–
Western Highlands	0	75	14	0	–	–	–	–	–	–
West New Guinea	–	–	–	–	–	–	17	1	82	22

tempting to implicate malaria as the selective agent, they point out that more information is needed on the possible role of factors such as filariasis, which is holoendemic also in the lowland areas and on the arbo-viruses, some of which are known to occur in lowland but not in highland populations. Finally, little is known of the tribal patterns which may have influenced the distribution of the Hp^1 gene, and which may have resulted in the observed distributions independently of the action of selective factors. This problem will be referred to again in discussing the distribution of G-6-PD deficiency and elevated Hb A_2 levels in New Guinea.

Connell et al. (1962) showed that the Hp^1 gene exists as two subtypes, Hp^{1S} and Hp^{1F}, and it may be expected that the frequency of these allelic genes will vary in different populations, an expectation which has been confirmed recently (Giblett and Brooks, 1963). Only one study has been made in either Australia or New Guinea of the distribution of these subtypes. Flory (1964) found 2 persons out of 102 in North Queensland with the Hp^{1F} gene. This gives a frequency of 1 per cent for the Hp^{1F} gene in this small heterogeneous sample.

Transferrins

Three transferrin variants, in addition to the common transferrin C, have been reported in Australia and New Guinea. One of these, which on starch-gel electrophoresis appears as a slow-moving D variant, has been found in all populations studied, whereas the other two, both of which are B variants, have a highly localised distribution.

The gene controlling the D transferrin (Tf^D) reaches its highest frequency in the Western Desert region of Australia. Here it has a value of 20 per cent; in the Kimberleys and Cape York, the Tf^D frequency is a little less than 10 per cent. Similar frequencies are found in New Guinea, where the Tf^D frequency ranges from 6 to 14 per cent. In contrast to the Hp^1 gene frequencies, there is no marked difference in the frequency of the Tf^D gene between highland and lowland populations (Curtain et al., 1964).

Kirk et al. (1964) have critically examined the D variants which occur in populations in various parts of the world. They have been unable to discriminate the D transferrin in Australia and New Guinea from the D_1 variant characteristic of African Negroes.

One of the B transferrin variants was found at Edward River in Cape York. This was described originally (Kirk et al., 1962) as being similar in mobility to B_1, but it appears to have a mobility intermediate between B_1 and B_2, but not identical with the B 1–2 variant found in

serum from a Venezuelan Indian (Arends et al., 1962). Flory (1964) has reported also a single person with a B variant of mobility between B_1 and B_2 in a series from north Queensland. The other B variant was described by Lai (1963) in a sample from near Lae in New Guinea. This variant B_{Lae} has the greatest mobility in starch-gel of all the transferrin variants. Curtain et al., (1964) have found further examples of this variant in the Bukawa linguistic group in the Markham River Valley, and one further example among the Yagwai of the Easter Highlands. Examples have been found also in persons belonging to four linguistic groups in New Britain. Curtain and his colleagues point out that the existence of B_{Lae} in the Tolai people of New Britain suggests a link between these and the people of the Markham River Valley. More detailed anthropological studies are needed of the distribution of B_{Lae} in this area. Curtain et al. (1964) have determined also the total iron-binding capacity for all the individuals in their series: they consider this to be a measure of the transferrin level. They find no significant difference in iron-binding capacity between the various geographic or linguistic groups included in their survey, or between the sexes or various age groups. The serum iron levels, however, are significantly lower in the 0–10 year age group in males compared with the 11–20 and older age groups. Female values of course are lower in the older age groups.

The Gc Groups

Using the technique of immuno-electrophoresis, Hirschfeld (1959) showed that variations in an α_2-globulin present in normal serum, the so-called group specific component, were inherited. The variations observed behaved in family studies as though they were under the control of a pair of codominant alleles, Gc^1 and Gc^2.

Other alleles have been described recently. One of these, $Gc^{Chippewa}$, has been found so far only in Chippewa Indians: another, $Gc^{Aborigine}$ (Gc^{Ab}) was discovered in Australian Aborigines. (Cleve et al., 1963).

In Australia the Gc^1 allele has a high frequency in all areas, exceeding 80 per cent except in Cape York. Here the value lies between 70 and 80 per cent, except for a small sample with a value of 90 per cent at Weipa. The Gc^{Ab} allele, which was discovered in a family at Aurukun in Cape York, has a very low frequency over the greater part of Australia. There is one focus in Central Australia where the Gc^{Ab} frequency is 5 per cent, and another in Cape York with a similar frequency of 5 per cent. In the Western Desert region, the Gc^{Ab} allele has disappeared almost completely. (Kirk et al., 1963a).

The only published investigation of the Gc groups in New Guinea

is for a small series of 45 persons from various places examined by Kirk et al. (1963b). Although, because of the small size of the series, the results of this study are of limited value, the frequency of the Gc^1 allele, 72 per cent, suggests that it may be lower in New Guinea than in Australia.

Among the small New Guinea series, seven persons carried the Gc^{Ab} allele, two of them in homozygous form. This suggests that the Gc^{Ab} frequency may be very high in some parts of New Guinea. It is of interest that this allele has not been detected in populations from other parts of the world, with the exception of another Melanesian population from the New Hebrides (Cleve and Kirk, unpublished observations). Recently further examples of Gc^{Ab} have been found in New Guinea and a detailed study of the distribution of the Gc groups in both highland and lowland populations is almost complete (Curtain and Baumgarten: personal communication). These workers report a wide range of frequencies (55–100 per cent) for Gc^1 in a preliminary study of populations in the Eastern Highlands. Further detailed results will be awaited with interest.

The Gamma Globulin Groups

The serology, genetics and world distribution of the Gm and Inv groups have been reviewed recently by Steinberg (1962). Detailed studies of the distribution of these groups in Australia have been published by Ropartz et al. (1962), and by Vos et al. (1963).

The Aborigines of the Central and Western Desert regions are unique in possessing no Gm (b+) persons: more than 600 persons have been tested so far. The frequency of Gm (b+) persons is also very low in Cape York (Steinberg and Kirk: unpublished observations). In contrast the Gm (a) character is universally present, the only exception being the single person report as Gm (a−) by Flory (1964 in a series from north Queensland.

In those populations where Gm (b) is present, it can be assigned to the postulated Gm^{ab} allele (Steinberg et al., 1960a). If the gene frequencies are calculated on this basis, Gm^{ab} is zero over the whole Central and Western Desert region and varies from zero to 5 per cent in Cape York and to 17 per cent in the Kimberleys.

The Gm(x) phenotype varies widely in frequency, having its lowest value at Mitchell River in Cape York (19 per cent) and its highest at Papunya in Central Australia (61 per cent). Gm(c), which is characteristic of African Negroes (Steinberg et al., 1960b) is absent in Australia.

The Inv groups have not been studied so intensively as have the Gm groups. In Australia, Inv(a+) phenotype frequencies are 49 per cent

in the Western Desert, 45 per cent in the Kimberleys and range between 32 and 61 per cent in Cape York and between 37 and 47 per cent in Central Australia. These figures for Australia suggest that the Inv groups are somewhat more stable than the Gm groups.

A limited number of populations have been studied for the Gm system in New Guinea. I am indebted to Drs. Gajdusek and Steinberg for permission to quote from their unpublished results. As in Australia, Gm (a) is universally present and Gm (c) universally absent. The Gm (b+) frequency ranges from 97 per cent in West New Guinea to 70 per cent for the Kukukuku in the Eastern Highlands and only 38 per cent for a sample of 37 persons in the Sepik River Valley. Gm (x+) frequencies vary in the opposite manner, being highest in the Eastern Highlands (15 per cent) and lowest in West New Guinea (2 per cent).

The Inv (a+) frequencies in New Guinea are among the lowest reported anywhere in the world. The value of 22 per cent in West New Guinea compares with values for Europeans, but the Kukukuku and Sepik River populations have the extremely low values of 7 and 5 per cent respectively. These values are in striking contrast to that of 75 per cent for another Melanesian population in the British Solomon Islands recorded also by Gajdusek and Steinberg.

Other Serum Protein and Enzyme Systems

Ag system. Allison and Blumberg (1961) described a precipitin system using serum from multiply transfused patients against normal serum on agar diffusion plates which distinguished two classes in the population:— Ag (+) and Ag (−). A small number of population studies have been carried out using this system which indicate that significant differences may exist between some populations in the incidence of Ag (+) persons (Allison and Blumberg, 1961; Blumberg et al., 1962). No New Guinea sera have been examined so far, and only a limited number of Australian sera have been tested. Dr. Blumberg has kindly made the results of these tests available. Approximately 84 per cent of Aborigines in Western Australia and Central Australia are Ag (+), a frequency intermediate between that of White and Negro Americans (54 and 55 per cent, respectively) and Micronesians (98 per cent).

Esterases. Two distinct variations in serum esterases are under genetic control, one resulting in suxamethonium sensitivity in persons homozygous for the recessive gene (Kalow and Genest, 1957), and the other demonstrable as an additional slower C_5 band after starch gel electrophoresis at pH 5–6. (Harris et al. 1963). The two variations are independent of one another.

The former variation reveals itself as an atypical enzyme in persons

heterozygous for the gene. Heterozygous persons have a frequency of roughly 3 per cent in European populations. Horsfall et al. (1963) examined 104 samples of serum from Aborigines in Queensland: only one person had the atypical enzyme. Whittaker, Harris and Kirk (unpublished) have examined 100 sera from Aborigines in Western Australia without finding a single person with the atypical enzyme.

Recently Curtain and his colleagues have examined a large number of sera from New Guinea using a system based on the rate of hydrolysis of butanylthiocholine measured with a silver-thiol electrode (Curtain et al., 1964). The frequency of heterozygotes, or persons with atypical enzyme varies from just under 1 to 4 per cent in various parts of New Guinea. No single case of a homozygous recessive person was found in nearly 2,000 serum samples tested (Curtain, personal communication).

The electrophoretic variation in serum esterase demonstrated by the presence or absence of the C_5 band, has not been studied in New Guinea populations. In Australia, unpublished studies by Kirk indicate a frequency for C_5 of less than 2 per cent in 166 sera tested from Australian Aborigines. Horsfall et al., (1963) found no C_5 variants among 104 samples from Aborigines in North Queensland. The C_5 band has a frequency of roughly 10 per cent in Europeans.

No studies of red cell esterases (Tashian, 1961) have yet been published for populations in New Guinea or Australia.

Other Biochemical Polymorphisms. No investigations have yet been published for Australia and New Guinea of the incidence of qualitative variations in the lactic dehydrogenases, 6-phosphogluconate dehydrogenase, red cell acid-phosphatases, serum alkaline phosphatases and catalase or for the rate of inactivation of isoniazid. There is evidence of polymorphic variation in these systems in at least some populations in the world.

Budtz-Olsen (1958) attempted to determine the level of excretion of mercaptan in the urine after ingestion of asparagus among Central Australian Aborigines, but was unable to determine the mercaptan levels under field conditions. No studies have been reported for the incidence of low or high level urinary excretion of β-amino isobutyric acid in Australia or New Guinea populations.

Sensory Polymorphisms

Ability to Taste P.T.C.

Simmons and his colleagues have used a simple method of testing for taste sensitivity using filter paper soaked in a solution of phenylthiocarbamide. Although the method cannot be regarded as giving accurate in-

formation, even more particularly when used for testing persons whose command of the language of communication is very limited, some data are available. They found 50 per cent tasters among 152 and 74 Aborigines at various places in the Northern Territory and at Haast's Bluff, respectively (Simmons et al., 1954; Simmons et al., 1957).

Simmons et al. (1961) have summarized the similar data available for New Guinea, pointing out that difficulties in interpretation were encountered and that the results were given with some doubts as to their reliability. At Mt. Hagen, 25 per cent were classified as strong tasters, 29 per cent as weak tasters and 46 per cent as nontasters. At Wissel Lakes, among the pygmies, nearly one-third could not be classified. In the Eastern Highlands, out of a small group of 30 tested more carefully, 77 per cent were classified as tasters, no distinction being made between the strong and weak classes.

Red-Green Color Blindness

Post (1962) has drawn attention to the fact that the incidence of red-green color blindness is very low in food gatherer-hunter communities, but higher in agricultural communities. He suggests that the difference is due to a relaxation of selection among agriculturalists. Mann and Turner (1956) using Ishihara charts, tested 286 Aborigines in the Kimberleys and found 2.5 per cent of the males and none of the females to be red-green color blind. Among 327 Aborigines in the Kalgoorlie area, mainly Western Desert natives, only 1 per cent of the males and none of the females were color blind. The frequencies in both surveys were significantly lower than among Europeans tested in the same localities at the same time.

In the same survey, Mann and Turner found 2 per cent of over 4,000 male Papuans to be red-green color blind, and 1 female out of nearly 3,000 female Papuans tested. The authors comment that the distribution of the red-green defect is uneven among the Papuans. For instance, in the Marshall Bennett Island the incidence among males is just over 4 per cent. W. H. R. Rivers, in a classical study of the Torres Strait Islanders (Rivers, 1901) found no color-blind males among 152 tested.

Hemoglobin Abnormalities and G-6-PD Deficiency

There is a marked contrast between populations in Australia and New Guinea in their experience of malaria. Although sporadic and highly localized epidemics of malaria have been reported in Northern Australia, they have always been self-limiting. In the coastal areas of New Guinea,

however, malaria is holoendemic and both *P. falciparum* and *P. vivax* are common (Kidson and Gorman, 1962a). The parasitemia rate falls with increasing altitude, and over great parts of the highlands the disease is absent.

Early investigations focussed attention on the search for the sickling phenomenon in blood films. Horsfall and Lehmann (1953) found no evidence of sickling in 57 Aborigines from North Queensland and similarly Simmons (1958) found no case of sickling in 230 full-blood Aborigines in Western Australia. Horsfall and Lehmann (1956) added to their earlier study by using paper electrophoresis to search for abnormal hemoglobins among 148 Aborigines in Queensland, and Budtz-Olsen (1958) examined a further 123 cases from North Queensland and 100 from Central Australia. Brain (unpublished results) has added a further series from Western Australia. No example of an abnormal hemoglobin has been found in any of these investigations. Budtz-Olsen, using agar gels, failed to reveal the presence of any cases with elevated levels of hemoglobin A_2.

In New Guinea, Swindler (1955) examined a small number of blood films for the sickling phenomenon. Walsh and Cotter (1955) examined 161 specimens from Port Moresby and 280 from Chimbu in the highlands, and Simmons et al. (1961) tested a further 50 samples. All three investigations gave negative results.

More recently, electrophoretic methods have revealed the presence of hemoglobinopathies in New Guinea. Ryan, Campbell and Brain (1961) described a case of hemoglobin H disease from Kerema, and Neeb et al. (1961) reported Hb_{Lepore} from Hollandia, on the north coast. Curtain et al. (1962) in a study of more than 1,000 persons in New Guinea found another case of Hb_{Lepore} and in the same paper they report an example of Hb E in a small group of samples from hospital patients in Port Moresby.

Although the frequency of abnormal hemoglobins, even among coastal populations in New Guinea, appears to be less than 1 per cent, thalassemia appears to be more prevalent. Ryan (1961) reported a case of thalassemia diagnosed on clinical signs, and Ryan (1962) lists a further four cases of thalassemia minor. Ryan (1963) has described three cases with bony changes characteristic of chronic hemolytic anemia.

Curtain et al., (1962) have carried out an intensive study to determine the incidence of persons with elevated Hb A_2 fractions. An Hb A_2 level exceeding 4 per cent was considered indicative of the thalassemia trait. Elevated Hb A_2 levels were found to vary from 0–25 per cent in different villages in the Sause linguistic group of the Sepik River District. In a

smaller sample of the Abelam linguistic group in the same area, the frequency of elevated Hb A_2 levels ranged from 1 to 8 per cent, the lower values occurring at the higher altitudes. No cases of elevated Hb A_2 levels were found in the Eastern Highlands (in 238 samples) but 2 persons out of 95 Enga in the Western Highlands had raised levels.

The absence of abnormal hemoglobins in Australia is paralleled by the absence of cases of erythrocyte G-6-PD deficiency. Budtz-Olsen and Kidson (1961) using a rapid screening method tested, with negative results, 435 males and 287 females from north Queensland, Central Australia and Western Australia. In New Guinea, on the other hand, the incidence of G-6-PD deficiency among males reaches nearly 30 per cent in some areas. Kidson (1961) found less than 1 per cent deficient males in the Eastern Highlands. Parsons and Ryan (1962) found higher values in coastal populations, and Gorman and Kidson (1962) have given a detailed analysis for several areas. In this latter study, the frequency of G-6-PD deficiency among males varies from 3 to 29 per cent in the Markham River Valley, but it was only 1 per cent in the Sause linguistic group of the Sepik River Valley, although this area is as malarious as the former. Among a small sample of the Abelam linguistic group in the Sepik River District, the value was 8 per cent. In the Eastern Highlands the values in the various linguistic groups were: Tairoro 1 per cent; Auyara 5 per cent; Gadsup, Awa, Kukukuku, Yate and North and South Fore, 0 per cent. Small samples in West New Guinea gave frequencies for deficient males of zero among the Mulia, and 8 and 17 per cent, respectively, in the coastal areas around Merauke and Asmat.

Gorman and Kidson (1962) point out that, in favor of the malaria hypothesis for the prevalence of G-6-PD deficiency, high frequencies of G-6-PD deficiency may occur in coastal populations, where malaria is holoendemic, but that so far high frequencies have not been observed in highland populations. Indeed, Kidson and Gorman (1962b) show that in the Markham River Valley there is a close correlation between altitude and decreasing incidence of G-6-PD deficiency, as one might expect if the malaria hypothesis is correct. However, Kidson and Gorman point out that in New Guinea there are a number of exceptions to the generalization that high frequencies of G-6-PD deficiency occur in areas where the malaria incidence is high also. Among the Sause in the Sepik River Valley, for example, only 3 out of 324 males were G-6-PD deficient, and in many villages no case of deficiency occurred. Kidson and Gorman question the validity of the malaria hypothesis as it relates to the incidence of G-6-PD deficiency. The striking cline in frequency which their own data demonstrates in the Markham River Valley may be due

simply, they claim, to the existence of a large gene pool in populations around Lae, where the deficiency among males approximates 30 per cent. This gene pool has diffused outwards into other linguistic groups along the Markham River, the dilution of the pool increasing with geographic distance. It may be coincidental that geographic distance here is also correlated with altitude.

In the Eastern Highlands, Kidson and Gorman show that G-6-PD deficiency has been found in two geographically contiguous and linguistically related groups only. They suggest that their data provides further evidence of the genetic heterogeneity of New Guinea populations.

Several different forms of G-6-PD deficiency have been identified. These forms differ principally in amount of enzyme activity present in the deficient male, in electrophoretic mobility and in lability (Motulsky, 1964; Porter et al., 1964). Parsons and Ryan (1962) found evidence to suggest that two different forms of G-6-PD deficiency, corresponding to the Negro and the Caucasian type, were present in New Guinea. Kidson and Gorman (1962a), however, found that the deficient males in their series were all of the Caucasian or "low" enzyme level type. They suggested that the "high" enzyme level type reported by Parsons and Ryan was an artefact due to the presence of markedly anemic patients among their 'deficient' series. It is known that during periods of acute hemolysis, enzyme levels in deficient persons may rise due to the presence of an increased number of young red cells, with a higher G-6-PD content. It must be noted, however, that Parsons and Ryan sampled areas different from those studied by Kidson and Gorman. Further, it seems highly probable that Kidson and Gorman included in their series persons whose anemia was as severe as those included by Parsons and Ryan.

Recently, Porter et al. (1964) have studied a series of 98 persons from New Guinea using starch gel electrophoresis. The mobility of the G-6-PD component of all but one of the 40 males tested was that of the B type. The single exception had a slightly slower G-6-PD component identified as a 'Baltimore' type. One of the females was possibly heterozygous for the 'Baltimore' type. This suggests that this variant should be tested for more intensively among New Guinea populations.

Kuru

The clinical description by Zigas and Gajdusek (1957) of a new syndrome known as 'Kuru' or 'trembling disease,' which occurs with high frequency in certain parts of the Eastern Highlands of New Guinea

has stimulated intensive research into the genetic structure of populations in that area.

So far more than 1,500 patients in whom the disease has been diagnosed have died since the investigations started. It is an acute degenerative disease of the central nervous system. It progresses rapidly after onset and death ensues generally within 1 year. Recovery after the neurological signs have become established is rare. Extensive and detailed studies of possible infective or toxic factors in the environment have so far yielded no clue to the etiology of Kuru. On the basis of the similarity of some of the pathological changes in Kuru to those manifest by sheep affected with 'scrapie,' it has been suggested that the immediate agent may be a latent virus, and work is in progress to test this hypothesis (Gajdusek, 1963).

Kuru is restricted to persons belonging to the cultural and linguistic group known as Fore, or to a limited number of adjacent linguistic groups related to the Fore through intermarriage. Isolated cases elsewhere in New Guinea, outside the Fore area, have occurred always in Fore-region natives working away from home. The disease accounts for the death of about 1 per cent annually of the population of about 35,000 in the entire Fore region. There is, however, an uneven spread of the disease within the region, so that in some small villages the annual death rate due to Kuru may be as high as 10 per cent. Kuru mainly affects mature females, but about one-fourth of all cases occurs in children of both sexes. Adult males are rarely affected. The differential death rate in adults produces severe social problems in the most badly affected areas.

Three cases of tremor syndromes with similarities to Kuru, and which may be clinical variants of the same disease have been described by Wilson et al. (1959) in the Western Highlands of New Guinea. No disease of a comparable kind has been observed among Australian Aborigines.

The restriction of the disease to one linguistic group and to its immediate neighbors, suggested to Zigas and Gajdusek in their initial investigation that genetic predisposing factors may be involved. Following this suggestion, Bennett, Rhodes and Robson (1958; 1959) showed that there was a family pattern in the distribution of the disease which was consistent with a single gene model.

The striking difference in the sex and age incidence of the disease was accounted for by Bennett and his colleagues by assuming that Kuru is under the control of a single autosomal gene K, dominant to its allelomorph k in females and recessive in males. Females of genotypes

KK and Kk are potential victims, the heterozygotes manifesting the disease of late onset, the homozygotes showing early onset. Only males of genotype KK will be affected and these cases will be of the early onset type.

Gajdusek (1963) questions the simple genetic hypothesis, while Bennett (1962) referring to his own hypothesis, indicates that Kuru could be maintained with high frequency only if there was a considerable selective advantage associated with the Kuru gene. No such advantage has yet been demonstrated. Recently, McArthur (1964) has re-examined the mortality rates from Kuru in two of the census divisions of the Eastern Highlands. She has smoothed the age curves to take account of the probable inaccuracies in the age-estimates, and when this is done the bimodality in the mortality and morbidity curves for females disappears. Instead, both the mortality and morbidity rates for females increase steadily with age to a maximum at about 45 years of age.

McArthur has estimated the frequency for the gene K from the life table for males in the South Fore census division, where mortality rates from the disease are highest. Her estimate is 0.45. A similar calculation based on the female life table yields a gene frequency for K of approximately 0.60. The difference between the estimates based on the male and female death rates is significant. A similar discrepancy occurs also in the Gimi census division, although in this case the values are considerably lower and the frequency based on the male mortality is higher than that based on the female (0.19 and 0.07, respectively).

McArthur points out that for the South Fore, the estimated gene frequencies are too high to be maintained in a population where the homozygous KK individuals die before reaching reproductive age. She concludes that if Kuru is genetically determined it seems unlikely to be through the mechanism postulated by Bennett and his colleagues. A similarly critical review of the genetic basis of Kuru has also been published by Williams et al. (1964).

Discussion

Genetic studies can contribute significant information to help solve a number of problems in human biology. These problems range from the delineation of the clear-cut genetic basis for specific diseases, including aberrations of the chromosome complement and the search for linkage relationships, to an assessment of the manner in which major and minor evolutionary changes have occurred within the human species. This latter application of genetic studies has caught the imagination of many

investigators, and all too frequently on the basis of inadequate information, theories have been advanced to explain the origins of particular human populations.

Studies in Australia and New Guinea during the last two decades have provided a substantial amount of genetic information at the purely descriptive level. The most general result which is apparent, is that for many polymorphic systems the range of gene frequencies between separate populations is very great. In some cases, e.g., the ABO blood group system, the range of frequencies is, or is nearly, as great as that for the rest of the world. In other cases, as for instance for the Ns chromosome, the frequency range is unique, setting off the peoples of either Australia or New Guinea distinctly from practically every other population which has been studied.

Within a narrower framework there are certain genetic markers which distinguish all Australian populations fom all New Guinea populations. In the serum protein groups there is no overlap at present between the frequencies of the Hp^1 gene or the Gc^1, Gm^a, Gm^{ax}, Gm^{ab} genes and the Inv (a+) phenotype. ← More adequate sampling of New Guinea populations, however, may well reveal overlaps for the Gc, Gm and Inv systems. For the blood group systems only the R^1 gene of the Rh system approaches the situation of nonoverlapping frequencies in the two areas.

In Australia, three genes show extreme values. The genes *mS* and *nS* of the MNSs system are zero over the entire continent and the gene *B* of the ABO system has a highly localized distribution, with a frequency of zero in most of the populations studied. In New Guinea both the frequencies of *mS* and *nS* are low and become zero in some places, but blood group *B* is always present.

Two qualitative similarities may be of greater significance than the quantitative comparison given above. An electrophoretically slow-moving transferrin variant, indistinguishable in mobility from the D_1 characteristic of African Negroes, is present in all populations studied in Australia and New Guinea. Elsewhere in the world, this variant has been reported, with the exception of some cases from Sweden, only in populations with probable Negro admixture in South America, or in other Melanesian populations (Kirk et al., 1964). It is possible that more refined technical methods may demonstrate that the D_1 variant in Africa is not identical with the D variant in Australia and New Guinea. This situation would be similar to the differences in amino-acid sequence found between some hemoglobin variants with identical electrophoretic mobilities (Baglioni, 1963).

Whatever may be the genetic relationship between Negroes and the

oceanic populations of Melanesia, it is tempting to postulate that the widespread distribution of this transferrin D variant in Australia and New Guinea indicates that at some time in the past there has been a common pool of genes between populations in at least these two areas. This postulate is strengthened by considering the distribution of the Gc^{Ab} allele. This allele, which is present in populations in New Guinea and Australia as well as in other parts of Melanesia (Cleve and Kirk, unpublished) has so far been found nowhere else in the world (Kirk et al. 1963b).

Within both Australia and New Guinea there exist clines as well as sharp discontinuities for gene frequencies between different populations. Birdsell (1950) and Birdsell as quoted by Dunn (1959) has drawn attention to this phenomenon in Australia. In his earlier work Birdsell attempted to demonstrate the pattern of gene flow in Australian Aborigines, using the known tribal distribution, and making assumptions about the resistance to gene flow at each tribal boundary. This work was handicapped by inadequate information on the distribution of genes in many parts of Australia, and these deficiencies are still acute today for many parts of the continent, in particular the densely populated Arnhem Land area.

The existence of sharp discontinuities may be illustrated by the existence of several populations in the Central and Western Desert regions of Australia in which the Gm (b) character is absent. The precise boundaries of this region are being mapped at present (Kirk et al., unpublished), but they appear to be coincident with the boundaries of a linguistically distinct group. To the west, north and east of this area Gm (b) is present, although in Cape York it has a low frequency. Since the time when the loss of this character occurred in the desert region, there appears to have been no significant gene flow into the population from neighboring groups. This latter observation is not concordant with that of Tindale (1953) who indicated that there are about 15 per cent of intertribal marriages per generation. Although this may be the case for the examples tested by Tindale's analysis, it is obvious, from the absence of Gm (b) over such a wide area, that some tribal boundaries are very much harder to cross than others.

In Australia, with the exception of the example just cited, no attempt has been made to correlate the distribution of genetic markers with the pattern of distribution of communalects or dialects. Another approach has been made by Simmons et al. (1962) to estimate the length of time for which a particular Aboriginal population may have been isolated. The Bentinck Islanders are an almost unique population with only blood

groups O and B present. The Bentinck Islanders are distinctive also in their dialect, in having no subsection system in their social organization and in not possessing the dingo. A consideration of the eustatic changes in sea level since the end of the Pleistocene suggested that the isolation of the Bentinck Islanders from their mainland ancestors could have been no earlier than 6,100 years ago, and it may have commenced no longer than 3,500 years ago. The authors suggested that this population probably reached the island by raft at about this time, and that its unique gene frequencies were produced largely by random genetic drift, most significantly in the original migrant population.

Linguistic information has been used by Watson et al. (1961) in an attempt to correlate the distribution in New Guinea Highlands of a highly localized D^u variant to the closeness of tribal relationship indicated by the glotto-chronology and the topography of the tribal areas. Livingstone (1963) in a critical paper uses the same data together with additional material for other highland populations in the same region to demonstrate that there is no correlation between the frequency of various genetic markers and degree of relationship indicated by the percentage of cognates in the languages.

Livingstone's paper makes clear that no simple study of gene frequencies is likely, by itself, to supply valid information on ancestral relationship between populations belonging to different linguistic groups. Such information needs to be supplemented by detailed understanding of the cultural barriers promoting or inhibiting gene flow between, or altering differentially the genetic structure of the populations concerned. Practices such as marriage by capture, polygamy, or establishment of trading relations in certain directions but not in others, may all lead to changes in genetic structure at rates not paralleled necessarily by changes in language. Stimulated by the problems presented by the disease, Kuru, the beginning of such detailed studies has been made for the Eastern Highland populations in New Guinea.

A close examination of the existing information on the distribution of genetic markers in various populations shows that many gaps remain to be filled, not only at the descriptive level but also at the level of interpretation.

Differences between populations may arise from mutation, selection, random genetic drift, or combinations of these. New mutations provide valuable tools for charting the spread of genes, if adequate genealogical records can be constructed for the populations concerned. Two examples of such mutants are the B transferrin variant in Cape York and the transferrin variant B_{Lae} in New Guinea, both of which probably estab-

lished themselves in the population relatively recently. In the case of B_{Lae} it is known to occur also in New Britain and it should not be impossible to determine its most probable site of origin and its pattern of dissemination.

The relative contribution of selection and genetic drift in producing significant differences in gene frequencies between closely related populations is likely to be difficult, if not impossible, to assess. Though the implication of selective agencies like malaria is suggestive for controlling the distribution of certain genetic markers such as G-6-PD deficiency, there is at present not even a suggestion of what selective factors may be involved in maintaining the other genetic polymorphisms such as variations in transferrins, haptoglobins or many of the blood group systems. Moreover, the attempt to determine such selective factors through studies on small indigenous populations is beset with difficulties (c.f. Neel et al., 1964). Even for a character such as G-6-PD deficiency, where there is a *prima facie* case for its frequency being maintained through the selective agency of a malarious environment, the work of Kidson and his colleagues outlined above indicates that other important factors have to be examined to account for the observed distribution in New Guinea.

In recent years, many workers have given greater weight to genetic drift in producing differences in gene frequencies between related populations of small size (e.g., Birdsell, as quoted by Dunn, 1959; Simmons et al., 1961). If genetic drift in addition to random sampling of the gene pool at the time of mating is taken to include the sum of events such as flood, epidemic disease, war and famine, which may remove by chance unequal proportions of the phenotypes present in a given population, as well as social factors which may give accidental preference to the spread of one gene rather than another, as in the case of polygamy restricted to a chief, then clearly drift can be envisaged as playing a significant part in producing genetic divergence in small human breeding isolates. Finally, it is important to recall the caution expressed by Cavalli-Sforza (1963) that, in implicating drift as a major cause for observed differences between populations, we have to be certain that historical accidents or geographical heterogeneity in selective conditions have not contributed significantly.

Little precise information is available for the effective size of breeding isolates in New Guinea populations. In Australia the average size of the tribe is generally considered to have been 500 persons (Kryzwicki, 1934; Birdsell, 1957) but this was frequently composed of a number of hordes of smaller size which formed separate breeding isolates in many

cases. Here again the evidence on the effective size of these isolates is inadequate. It should be noted, moreover, that in both New Guinea and Australia no studies have been published on the amount of inbreeding present in the indigenous populations.

However, despite these limitations, enough information is available on the social structure of tribal populations to make feasible the machine simulation of the effects of drift and selection over a large number of generations in units of comparable structure. A stimulating example of the potential usefulness of such an approach has been provided recently by Brues (1963), and the further exploration of these techniques will be awaited with interest.

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CHAPTER 8

Twin Research: Problems and Prospects

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THE USEFULNESS OF TWINS in medical research is very limited and specialized. Growing recognition of this fact and mounting successes with other research methods have left twin investigators isolated if not beleagured. Twin research of the past has answered a variety of simple, often naive, questions and has provided some crude estimates of the importance of environment in determining human traits and diseases. Many remaining problems that might be solved with the aid of twins will require precision or sophistication rarely achieved hitherto. This is not to say that students of twins have been satisfied with answering simple questions, or that twin studies cannot be good and simple at the same time. Twin studies have generally fallen short of their designers' objectives because of unsuspected complexities in the biology of twinning, or because of unsuitable experimental design and incorrect interpretation. Even the majority of successful twin studies have led into blind alleys because they did not seek the kind of information that other scientists could build upon.

The immediate object of most twin studies is to evaluate differences between identical or monozygotic twins, and the most convenient if not the best control material is usually found in fraternal or dizygotic twins. But the value of twins does not begin and end with the fact that some are genetically identical and some are not. Twin research is not so much a tool for genetic analysis as a means of testing hypotheses about environmental effects. Twins share a common prenatal environment and closely similar physical and social environments. Monozygotic (MZ) twins start life with the same parental cytoplasm as well as with the same genotype. For internal comparisons, a series of MZ twins can, ideally, be subdivided into types with different placental relationships, while dizygotic (DZ) twins can be separated into same-sex and op-

posite-sex types. DZ twins occasionally show the immunologically important phenomenon of chimerism due to early placental interchange of circulating cells. Finally, twins have parents and sibs that may yield information complementing or complemented by that from the twins. Thus twin studies offer unique approaches to a variety of problems not amenable to direct experimentation, but as indirect experimentation they should usually be designed to test specific hypotheses (Platt, 1964). For twin research to move in this direction, method-oriented twin specialists may have to relinquish initiative to problem-oriented scientists who are willing to learn twin methods.

Limitations of Twin Studies and Corrective Measures

Even carefully designed experiments may fail to control all relevant variables, and the phenomenon of twinning has many uncontrollable side effects. Some of these side effects can be measured or circumvented, others are as yet only suspected or known vaguely. In addition, twin research has its share of methodological pitfalls. In the following discussion space is allotted to topics as required for complete exposition and not according to relative importance.

The Multiplicity of Twin Types

Students of twins have traditionally disregarded warnings that there might be a third type of twins intermediate between the monozygotic and dizygotic in genetic dissimilarity. Some recent findings in individuals with two or more cell types (mosaics and chimeras) are presumptive evidence of irregular phenomena occurring at fertilization (Zuelzer, Beattie and Riesman, 1964). The same or related phenomena may sometimes result in twins. Irregular fertilization is probably rare, but twins are also somewhat rare and might include an appreciable proportion of abnormal zygotes. This is especially true if irregular fertilization proves to be associated with twinning, as has been suggested for Turner's syndrome (Nance and Uchida, 1964).

The more plausible hypotheses proposed for the origin of irregular twin zygotes all require two sperms, which would result in opposite-sex twins about half the time. The nonexistence or extreme rarity of opposite-sex *monochorionic* twins gives assurance that at least those pairs enclosed in a common chorion are nearly always derived from a single regular zygote (Juel-Nielsen, Nielsen and Hauge, 1958; cf. Pickering, 1946). The possibility remains that some dichorionic twins differ as much as sibs in the paternal chromosome complement while the ma-

TABLE 1—*Segregation of Single Loci between Twins Derived from an Egg and a Polar Body*

Identical daughter genes from one chromosome are designated A and A′; those from the homologous chromosome a and a′.

	Products of the First Meiotic Division		Twins from Egg and First Polar Body				Twins from Egg and Second Polar Body			
				Twin 1	Twin 2	Concordance		Twin 1	Twin 2	Concordance
Proximal loci	AA′	aa′		A	a					
			or	A	a′	0		A	A′	100%
			or	A′	a		or	a	a′	
			or	A′	a′					
	AA′	aa′		same as above		0		same as above		100%
Distal loci				A	A′					
	Aa	A′a′	or	A	a′	50%		A	a	0
			or	a	A′		or	A′	a′	
			or	a	a′					
				A	A′					
	Aa′	A′a	or	A	a	50%		A	a′	
			or	a′	A′		or	A′	a	0
			or	a′	a					

ternal complement is derived from one meiotic event. The maternal cells might be two identical blastomeres, an egg and a large first polar body each undergoing the final meiotic division after fertilization (Mijsberg, 1957), or an egg and a second polar body (Lehmann and Huber, 1944).

Twins derived from two sperms and two blastomeres would have half as many differences as ordinary siblings, but these would presumably be enough to destroy identity of the genetic background, the most distinctive genetic property of monozygotic twins. Ordinary objective tests of zygosity would usually classify such pairs as dizygotic. Since DZ twins are by no means uniformly dissimilar, inclusion of a few uniovuler, dispermatic pairs would be of consequence only in a very precise analysis of genetic and environmental components of variation.

If the two maternal cells were egg and a polar body they would never approach the similarity of blastomeres, but they might differ a little more or a little less than two eggs. With respect to a small proportion of their genes, differences could be very much more or less frequent, depending on cross-over distance from the centromere and depending on which polar body was fertilized. This problem has been analyzed by Rosin (1947); although he ignored the possible role of the first polar body, he made nearly equivalent calculations in allowing for possible postreduction of the centromeres. Table 1 illustrates the two

limiting cases for heterozygous loci; those very close to the centromere and those at the ends of long chromosome arms with multiple chiasmata. Sister centromeres are assumed to separate in the second division. If a distal locus segregates at random, one of four homologous genes at meiotic prophase has equal chances of being paired with any of the other three, and the locus would be dissimilar in two-thirds of either type of polar-body twins.

By Rosin's calculations, loci within about 47 cross-over units of a centromere would be predominantly similar in second-polar body twins. If the estimate of human chiasma frequencies of Ford and Hamerton (1956) can be applied to the observed distribution of arm lengths of somatic chromosomes, as many as 65 per cent of the genes may be within 47 cross-over units of a centromere. This would determine a slight preponderance of similar loci in second-polar body twins. First-polar body twins would have considerably fewer similarities than sibs. The discrepancies would be only statistical, best discerned by analysis of many loci in many twin pairs (Mijsberg, 1957). But if a number of loci were known to be close to a centromere, these would permit individual recognition of polar-body twin pairs among dizygotic twins.

If polar-body twins can be neglected, some other inaccuracies of a dichotomous classification of twins cannot. One irregular type that is certain to occur, even if rare, is the monozygotic pair differentiated by mutation or by chromosomal aberration originating in the embryo (Turpin et al., 1961; Mikkelsen, Frøland and Ellebjerg, 1963). The frequency of gene-mutation in the first three or four cell divisions is presumably small, but this may be the most likely time for somatic chromosomal aberrations (Lejeune, 1964, pp. 163–164). Cytological studies are therefore of value in any case of unusual disparity between apparently monozygotic twins.

Exchange of blood through early placental anastomoses produces chimerism in some pairs of dizygotic twins. This is not known to involve tissues outside of the hematopoietic system, but is important in immunological studies and can, of course, complicate the laboratory diagnosis of zygosity (Woodruff and Lennox, 1959; Kuhns, 1964). Until the frequency of such chimeras is known to be negligible, or until they can be faithfully detected, they must be regarded as a potential source of error in hematological and immunological data.

Related to the problem of other types of twins and numerically more important is the inhomogenity within the two main types. The placental differences among monozygotics require more study before their relevance can be assessed in any particular problem. Not only do the

membranes record differences in time and method of division of the embryonic cell mass, but also the extraembryonic circulation of monochorionic twins is always somewhat abnormal. Associated with these interpair differences are even more marked intrapair differences (see following section). Among dizygotic twins, opposite-sex pairs may be very different physiologically and socially from the same-sex pairs. Degrees of difference in postnatal experience are a major variable for twins of all types. Study of these differences sometimes yields critical information, for example as between concordant and discordant MZ pairs, but in general the inhomogeneity of each twin type complicates twin research and even restricts the possible objectives.

Differences between Twins and Nontwins

Twins can substitute for human experimentation only to the extent that they resemble the general population. Any general conclusions depend either on evidence that abnormalities in twins do not affect the observations, or on corrections that compensate for the abnormalities.

Of first importance, when any pathological condition is studied in twins, is the search for differences from the condition as found in nontwins, either in frequency or in form. Large differences can be attributed to direct effects of phenomena peculiar to twins or to one type of twins. If monozygotics are particularly susceptible, further study may locate the susceptibility in monochorionic twins. The finding that a condition is concentrated in twins may provide a clue to the etiology in nontwins, but the clue may be indirect.

Pathology in twins may be modified first of all by the etiological factors of twinning itself. Twinning is produced in animal experiments by teratogenic agents, and if monozygotic twins result from overripeness of the egg, genetic defect, or other abnormality of the early embryo, they may be prone to certain malformations (Strupler, 1947; Witschi, 1952). Similarly, dizygotic twinning may reflect a level of ovarian dysfunction in the mother that could cause pathology. Evidence has not been produced either for or against these hypotheses.

Second in time as possible causes of twin abnormality are early embryological *consequences* of identical twinning. Lateral inversion (mirror imaging) was once thought from experimental studies to be important in MZ twins. Human studies have failed to support this expectation (see Torgersen, 1950), but further systematic studies are needed. More important is the common placental circulation of monochorionic twins, constituting about two-thirds of MZ pairs. This and related subjects were reviewed by Price (1950). Imbalance in the

common circulation may produce any degree of defect in one twin, possibly in both. A small but long-continued arteriovenous flow from one twin to the other could produce severe imbalance in blood cell mass, plasma proteins, and probably blood-born nutrients (Klooster-man, 1963). The apparent relation of low birth weight in one of MZ twins to susceptibility of that twin to schizophrenia (Pollin, Stabenau and Tupin, 1965) or severe mental defect (Allen and Kallmann, 1962) may illustrate the ultimate importance of these circulatory phenomena.

A placental circulation that was balanced prior to onset of labor may become unbalanced during delivery, producing some cases of the "placental transfusion syndrome" (Falkner, Banik and Westland, 1962). A related phenomenon, differential exsanguination of the two placentas, usually favoring the second-born twin, may occur in dichorionic and dizygotic twins as well as in monochorionic pairs (Seip, 1956). The latter phenomenon is not potentially as serious as transplacental trans-fusion and does not differentiate twins from others because single infants also receive varying amounts of blood from the placenta.

In order to evaluate the consequences of circulatory imbalance in its different forms and degrees, there is a need for some careful follow-up studies of twins after collection of detailed birth data. These should probably include histology of the membranes, injection of the common circulation (Benirschke, 1961), serum biochemical studies, serial hemo-globin determinations and reticulocyte counts (Clemetson, 1956). Some of these variables have little relation to birth weight and would help to differentiate chronic from acute placental transfusion. Such knowl-edge can be applied in subsequent twin studies when description of twin placentas becomes routine obstetrical practice, a rather distant prospect.

The observation that MZ twins have lower average birth weight and greater birth weight differences than DZ twins might be due either to the common circulation or to division of the early embryo, and one of these alternatives might be eliminated by further studies of birth weight and placental form. *Monoamniotic* twins may be especially sub-ject to pathology because of very late separation or because they share a single amniotic space, but unbiased statistics will be difficult to obtain because these twins are relatively rare (Potter, 1963).

Other direct causes of pathology are shared by all twins, and to a degree by all babies. These include crowding during the last half of gestation, premature delivery, and mechanical abnormalities of de-livery; also frequent erythroblastosis and maternal toxemia. Several of these agents may lead to trauma, making cerebral palsy at least twice

as frequent in twins as in single births (Russell, 1961) and probably causing milder brain damage in some twins. Immaturity of most twins complicates the already difficult delivery of two fetuses, although twins are usually more mature than single prematures of the same weight. As might be expected on the basis of their prematurity, twins show an increased incidence of hyaline membrane disease and neonatal infection (Potter, 1963).

After birth, influences that may render twins atypical of the population are important mainly for psychological studies. The social and psychological impact of having a twin is unlike anything found in an ordinary sib relationship (Burlingham, 1952). Other postnatal differentiating factors are effective only by virtue of their greater frequency or magnitude in twins. Examples would be nutritional and physical privations that twins may encounter because of their extra demands upon mother and family. This may increase the tendency of parents to institutionalize twins, as in the case of mental deficiency (Allen and Kallmann, 1962).

When a condition shows only a small difference in frequency or form between twins and nontwins, indirect effects may be suspected. Maternal age, parity and race are related to frequency and type of twinning (Strandskov and Edelen, 1946; McArthur, 1954; Inouye, 1957), and therefore have a peculiar distribution in twins. Control of such interfering variables may be possible, when necessary, by matching techniques, by comparison of twins and single-born in appropriate groupings, or by the methods of analysis of variance.

Another way to circumvent the abnormalities of twins in statistical analysis is to use internal controls. If a twin series includes some conditions of known etiology, internal comparisons are often more informative than comparisons with nontwins. The usual comparison between MZ and DZ twins is an example of internal control, but requires assumptions about the factors that differentiate the two types of twins.

On a more positive note, the abnormal circumstances surrounding twins sometimes increase their research value. Twins provide a test of any theory explaining prematurity and its consequences, partly because the distribution of birth weights by gestation age is atypical. Again, one psychodynamic explanation of schizophrenia is based upon "confusion of ego identity." The absence of an increased frequency of schizophrenia in MZ twins throws doubt on the theory, and suggests that in any event some types of confusion of identity are not causative (Rosenthal, 1960). These are examples of how twins may be used to exclude specific hypotheses.

TABLE 2—*Types of Twin Occurrence Likely to Elucidate Diseases of Unknown Etiology*

	Monozygotic	Dizygotic
Concordant pairs	Pairs that show significant differences	All pairs
Discordant pairs	All pairs	Cases with pathology present from birth

Biases in Twin Samples

The special problems of bias in twin studies are not greater than ordinary sampling problems, but are usually superimposed upon the latter. It is difficult to detect all twins in a general population either of normal people or of patients with a given condition. This is especially true if the attempt is made, as it usually ought to be, to include persons who lost a twin partner in infancy. When clinic personnel start to ask every new admission if he is a twin, they receive so few positive responses that they soon become careless, and the only twins they are sure to detect are those that come in pairs. One remedy is to make the twin question one of a series that will usually elicit one or more positive responses.

At this point it will help to classify clinical studies into four familiar types according to mode and completeness of detection or "ascertainment."

(1) Case reports: A single pair of twins, very carefully studied, may be useful in showing what *can* occur. The types of case worth reporting, if similar cases have not already appeared in the literature, are indicated in Table 2. Multiple reports may be valuable if they illustrate differences, but representativeness in case reports is an unattainable objective.

(2) Collected case reports: Perhaps because of popular interest, case reports involving twins are published with little regard for their theoretical value or for the number of similar cases already known. The collection and analysis of these reports as if they described a valid sample gives the impression of statistical weight, but even if editors accepted every case report they received, distortion would occur, usually favoring the "all pairs" cells in Table 2. Only when reports accumulate in the other direction are they likely to show what usually happens. For example, the literature on twins with 21-trisomy (mongolism) has long reflected the now understandable fact that concordance is the rule in MZ twins and very unusual in DZ twins.

Twin studies that depend on self-reporting by twins or on voluntary

reporting by physicians suffer from the same deficiencies as collected case reports, if not quite to the same degree. Since the source population cannot be defined, no statistical control or correction is possible.

(3) Consecutive series: The most successful medical twin studies in the past have been those that found twins in a defined population of clinic or hospital patients. The prerequisite for a series is the decision to report them before any are observed, or the exclusion of cases seen before the decision was reached. This approach does not automatically eliminate the biases of single case reports, but a reference population permits one to recognize and deal with possible bias. Undesirable biases arise in consecutive series from failure to include all cases that belong to the defined patient population and from discrepancies between the defined population and other affected persons. In the limiting case, where all affected individuals are detected in a general population, the problems of truncate selection remain, that is, unaffected susceptible persons go unrecognized.

(4) Total ascertainment of a twin population: This method avoids the most serious sampling biases, but it requires time and expense that would usually be out of proportion to suitably specific objectives. A sizeable population may contain just one or two twin cases of each of several rare conditions; the selection of some instances to report is then open to the same objections as a simple case report.

Consecutive series of patients will probably continue to provide the bulk of material for future medical twin studies. When the object is to study concordant twin pairs, or when qualitative conclusions will suffice, biased ascertainment may be harmless or even helpful. When statistics are desired, such as concordance rates, the inevitable omission of some twins calls for statistical control by at least three criteria. First, the frequency of detected twins in the series should be as high as in the general population, if not higher. However, the usual twin frequency may not apply, and such variables as age, maternal age, and race may need to be considered. Second, the proportion of affected individuals (not pairs; see section on estimation of zygosity proportions) representing same-sex and opposite-sex twins should agree with that for the appropriate population, and here again age and race may be relevant. For example, recent data on twin births and survival would be misleading if compared with twin frequencies among adult cancer patients. Third, frequency of detection of twins should be nearly uniform throughout the study within statistical expectation. If some portions of the sample appear to reflect poor screening for twins, these portions can be analyzed separately to estimate the consequences of incomplete ascertainment in

the entire series. It is unfortunately true that most twin series are so short that these statistical controls detect only very gross biases.

Detection of twins already residing in an institution is subject to more errors than detection of consecutive admissions. Unless every case record is examined and includes a list of the patient's sibs, ease of detection will favor concordant over discordant pairs. The other and more general source of error is variation in length of stay. This is likely to depend on concordance in twins, as well as on severity and type of illness, so that the sample will not represent the population of affected twins, either those outside the institution or those admitted. These biases may be revealed in an excess of individuals from same-sex pairs. If the excess is small, some conclusions from the relative concordance rates may still be valid, since the concordance rate of DZ twins will be increased along with that of MZ twins.

Total ascertainment of twin populations is becoming more frequent and more successful. Some studies start with a series of recent births and attempt to follow twins through the age period of interest. Adult studies have been based on military service records checked against birth records, or on a series of old birth records that could be traced to living subjects. There is always considerable loss of cases in this process, but the losses are assumed, or sometimes can be shown, not to bear upon the results.

The above discussion pertains mainly to discrete pathological conditions. Continuous variables like stature or blood pressure are usually studied in a normal sample. The use of self-reporting twins in such a study prevents any general conclusions about the entire twin population, but so does the use of a specialized sample such as twins in an institution. No study of twins permits precise quantitative conclusions about the nontwin population, so that great effort to secure a complete or representative sample is rather futile.

In summary, sampling biases must always be assumed in twin studies. Only qualitative conclusions are likely to have general validity, although quantitative conclusions about the twins themselves may be useful in constructing or excluding general hypotheses.

Estimation of Zygosity Proportions

Of major importance in almost any twin research is classification of the twins by type, either individually or collectively, and this is one of the commonest bases of criticism. Of course, if twins are all discordant or all concordant for a condition under study, the only reason for classification is to assure that both types are represented. When relative con-

TABLE 3—*Demonstration of Weinberg's Difference Method with Heterogeneous Data*

Twins in 40,000 deliveries of four equal groups of hypothetical mothers

	MZ Twins	DZ Twins		Total Same-sex
		Same-sex	Opposite-sex	
Mothers A	30	30	30	60
Mothers B	30	30	30	60
Mothers C	40	80	80	120
Mothers D	60	40	40	100
Totals	160	180	180	340

Estimated MZ twins: 340 − 180 = 160

cordance rates in monozygotic and dizygotic twins are desired, comparison of same-sex and opposite-sex pairs may indicate the direction of the difference, and, with the aid of Weinberg's differential method (see below) even its magnitude. This is the only approach possible when pairs cannot be classified individually, as in vital statistics. The differential method is useful in other ways, and in fact is basic for the statistical study of twin samples.

The differential or difference method (Weinberg, 1901) estimates the number of MZ twins in a sample as the difference between same-sex and opposite-sex classes. In this simple form, the method assumes only that these classes are equal in DZ twins alone. This assumption is essentially correct in a variety of circumstances where use of the differential method has been criticized. For example, variation in twinning rate between races or maternal age groups within a series does not affect the method, as the exaggerated example in Table 3 shows.

Caution must be used, however, in calculating the ratio of types in a twin population for comparison with a twin sample. Significant errors may sometimes result if the sample is from a different population, or from a segment of the population having an atypical distribution of maternal ages and parities. The differential method should probably not be used at all with a twin series collected by voluntary reporting; the estimate of DZ twins will be too low if the reporting sources are less interested in opposite-sex pairs. Thus, Osborne and DeGeorge (1959) found only 30 instead of 50 per cent of male-female pairs among their DZ twins. This kind of bias may be correlated with a disturbance of the over-all sex ratio; when an observed inequality of the sexes is not readily explained on the basis of population sex ratio or disease incidence, it should serve as a warning against use of Weinberg's difference method.

When an unbalanced sex ratio is to be expected, a question arises

TABLE 4—*Demonstration of Weinberg's Difference Method in Case of a Disease with Different Incidence in the Sexes*

Morbidity rate .005 in males, .001 in females

	Monozygotic		Dizygotic		
All twin pairs	10,000		16,000		
	MM	FF	MM	MF	FF
	5000	5000	4000	8000	4000
All twin individuals:					
Male	10,000		8000	8000	
Female		10,000		8000	8000
Affected twin individuals	50	10	40	48	8

Estimated MZ twins. 108 − 48 = 60

about correcting the differential estimate. While discounting the need for it, Weinberg proposed a correction that is the same as that found with the maximum likelihood method by Gittelsohn and Milham (1964), except that these authors use the sex ratio in the twin sample. If the proportion of males is p and the proportion of females is q, so that $p + q = 1$, then dizygotic twins should occur as follows:

$$\overline{MM} \text{ (same-sex, male)} = p^2$$
$$\overline{FF} \text{ (same-sex, female)} = q^2$$
$$\overline{MF} \text{ (opposite-sex)} = 2pq$$

The total of DZ twins is then estimated to be the number or per cent of opposite-sex pairs in the sample divided by 2pq: $\overline{DZ} = \overline{MF}/2pq$. This estimate coincides with the simple difference method when 2pq equals 0.5: $\overline{DZ} = 2\,\overline{MF}$.

There are at least three sources of difficulty in the application of this formula. First, the values of p and q should be understood to represent frequencies of the two sexes in the normal twin population, not sex-specific morbidity rates. If one collected twins affected by a disease that occurred in 0.5 per cent of males and in 0.1 per cent of females, the zygosity types in affected twins would occur in the usual proportions, but an estimate based on the sex ratio among patients would suggest an aberrant distribution. In the example shown (Table 4), calculations from the sex ratio in patients would force one to conclude that there were only about six MZ twins in the sample. Note that the calculations are based on twin individuals (index cases; see section on problems of analysis); the use of pairs would yield nonsense without

knowledge of the concordance rates for each type of twins, usually to be determined in the study. This last precaution is unnecessary when the number of pairs in every category is half the number of individuals.

A second difficulty is the source of the sex ratio. Data from the general population or even from the twin sample itself are likely to be inappropriate for the dizygotic twins to which the sex ratio must be applied (see survival data of Record, Gibson and McKeown, 1952). The correct sex ratio is known only after detailed zygosity classification. Furthermore, differential survival of the sexes affects the estimates of zygosity types only when estimates are based on intact twin pairs. If individual survivors can be discovered and included, and if estimates are based on individuals as in Table 4, the relevant sex ratio is that prior to detectable mortality.

Third, factors affecting the sex ratio are likely to be correlated within DZ twin pairs. Sex at conception is probably not strongly correlated, but subsequent survival is an important determinant of the sex ratio and Karn (1953) found correlations in perinatal mortality of 0.56 in same-sex, 0.41 in opposite-sex twin pairs. Heterogeneity in the sex ratio, studied by Gittelsohn and Milham (1964), would also contribute to the correlation coefficient, though not important by itself. Such correlations introduce errors of the same order of magnitude as the sex ratio correction, since perfect correlation would eliminate from the estimate all effects of a sex difference in survival.

There is one further objection to the sex ratio correction. As has been repeatedly pointed out, and first by Weinberg himself, the magnitude of the correction is generally trivial. With a sex ratio as extreme as 80 males to 100 females, the difference method comes within one per cent of the proportions calculated from the sex frequencies. This would be about three per cent of the proportion of MZ twins, and as large as one standard error only in samples greater than about 3,000 twin pairs.

For the above reasons, use of the sex ratio in estimating zygosity proportions is sometimes quite wrong and at best is like reporting in five decimal places data accurate only to two. When an estimate is needed of MZ twins by sex, apportioning the estimated MZ total according to the sex ratio among all the twins is as accurate as estimating the sexes separately.

When vital statistics of twins are studied, it is often desirable to estimate the separate contributions of MZ and DZ twins. On the assumption that DZ twins are alike in number and average characteristics, within sex, whether they belong to same-sex or to opposite-sex pairs, the difference method can provide useful first approximations (Gittelsohn

and Milham, 1964). This procedure is somewhat more vulnerable than estimation of type frequencies in a population of surviving twins, because sex differences are likely to be more important while still defying accurate correction.

Classification of Pairs by Zygosity

Determination of zygosity in individual twin pairs is by now a fairly reliable and well standardized procedure, but some deviations in method are legitimate, and new and better methods are still needed. The classical method, based on fetal membranes, affords one of the most valuable criteria when good observations are available. A monochorionic placenta provides the most definite evidence of monozygotic origin, and also may have important medical consequences as described above. Unfortunately, obstetrical records of twins rarely specify anything more than the number of placentas, and this is not much help in zygosity diagnosis. In the Chicago Lying-In Hospital Potter (1963) found more than half of the twins to have a single placenta, but only one-fifth were monochorionic. The other MZ twins, about 40 per cent as estimated by the difference method, had apparently the same proportion of separate and fused dichorionic placentas as did the DZ twins.

It is not difficult to identify the majority of DZ twins. Half are of opposite sex, and of the other half about 80 per cent are likely to differ in one or more of the major blood antigens (Sutton, Clark and Schull, 1955; see male-female pairs of Potter, 1963). Somewhat less reliable blood antigens and serum differences are also useful (Juel-Nielsen et al., 1958) and their number is continually increasing. However, the availability of antisera or of laboratories able to perform the tests seriously limits their application, especially with large numbers of twins. Blood tests of newborns usually present special problems. With all blood testing there is some danger in accepting a single observed difference to identify a DZ pair, and Osborne (1958) found it advisable to have all tests done in duplicate. The more expensive tests need be applied only when the usual tests fail to detect a difference. Blood group determinations are so much more effective in zygosity diagnosis than other criteria that they should be routine for twin research until and unless other methods can be greatly improved.

Other methods are valuable in identifying the remaining DZ twins among the like-sexed pairs; for a full discussion the reader is referred to Dencker, Hauge, Kaij and Nielsen (1961). It is possible to distinguish five approaches that differ in usefulness and reliability, as follows:

(1) The simplest and doubtless the oldest way to classify twins is from their appearance. It takes very little experience to diagnose most pairs by casual inspection, and even parents and friends of twins probably do it unconsciously if not consciously. Cederlöf, Friberg, Jonsson and Kaij (1961) obtained apparently good results with mailed questionnaires. It would be foolish to disdain such methods altogether; in normal twins physical appearance can at least serve to raise suspicion of error in objective methods of diagnosis, while concordance for a rare anomaly helps to confirm monozygosity. The method should be eschewed or used with great caution in studies of conditions that affect the general appearance or behavior. For research purposes the criteria are too subjective to yield a record that others can review; even good photographs may be deceptive.

(2) A second auxiliary method achieves greater objectivity by emphasizing differences instead of similarities. Clearly, any good evidence of a genetic difference is evidence that twins are dizygotic. Hair or eye color or iris pattern may provide reliable differences, but they are difficult to quantify, to evaluate, or even to record. Any difference that can be measured is best judged against experience with other twins, and the method then merges with the next.

(3) Probability calculations can yield affirmative evidence of monozygosity, supplementing that from fetal membranes and from striking similarities (method 1 above). The likelihood that DZ twins should be concordant for a blood group can be calculated from Mendelian principles, although the possibility of selective survival makes empirical validation of the probabilities desirable. Empirical data on quantitative traits permit the estimation of relative likelihood that an observed difference should occur in MZ or DZ twins. Calculations outlined by Smith and Penrose (1955) may be applied to any character that has been recorded in a series of twins reliably classified by other criteria. Unless accurate information is available about the fetal membranes, this method should now be regarded as mandatory for acceptance of a monozygotic diagnosis in the study of one or a few pairs. In application to larger series some risks and systematic errors reduce the value of the method. To begin with, if the tests employed are expected to recognize differences in 95 per cent of DZ twins, the remaining 5 per cent of DZ pairs are likely to be classified as MZ within the 5 per cent confidence interval. Second, selective survival of DZ twins with respect to biochemical characters (Osborne and DeGeorge, 1957) may increase their similarity over that calculated from Mendelian theory, and undetected blood-group chimerism may hide some antigenic differences. Only empirical validation of

theoretical expectations can remove this source of uncertainty. Third, the calculations for simple traits depend either on knowledge of the parental genotypes or on estimates of gene frequencies in the appropriate population, which are often not available. Fourth, calculations for complex or quantitative traits depend on assumptions of independence which are often not justified. All of these sources of error excepting the third have the effect of placing DZ twins among the MZ.

(4) Generally as a last resort in difficult zygosity problems, tissue transplantation has been tried. The behavior of leukocytes in mixed cultures (Bain and Lowenstein, 1964) affords an easier immunological procedure, although compatibility between many dizygotic pairs is to be expected. Even when direct reciprocal transplants are successful the possibility remains of undetected chimerism in a DZ pair, and failure in at least one of an *identical* pair might result from an early somatic mutation that formed a protein foreign to the partner. Hence, this method also requires empirical validation in future studies; the resulting information will have practical and theoretical importance.

(5) The most rigorous approach to the zygosity problem is to label as DZ only those pairs showing simple Mendelian differences. This has the great merit of making only one kind of error. The number of apparently DZ pairs placed in the MZ category affords an estimate of the magnitude of the error, but the confidence level remains subjective with the investigator. Some investigators have recognized a category of undiagnosable pairs, which can be grouped first with the MZ and second with the DZ twins in duplicate analyses of the data.

Occasional errors in zygosity determination will probably always plague twin studies. The investigator should attempt to minimize these errors, but must also make allowance for them.

Miscellaneous Problems of Design: Diagnostic Bias, Triplets, and Siblings

The use of subjective criteria in zygosity determination is particularly to be avoided when these criteria may be influenced by the traits under study. A related problem is the danger that medical diagnosis may be influenced by extraneous resemblances between identical twins; a physician seeing the second member of a pair of MZ twins may recognize the face and voice and consciously or unconsciously recall the diagnosis of the first twin. This danger dictates the use of objective criteria as far as possible in medical diagnosis of twins. Where subjective decisions are necessary, the partners should be seen at widely separated times or by different examiners. Evaluation by different investiga-

tors permits uniformity with respect to pairs, if not with respect to individuals. The amount of bias introduced or the reliability added by letting each judge also see the other twin could be evaluated in the same study if the order of the twins was reversed for the two judges. Even with two separate examiners, some of their diagnoses or judgments may coincide because of extraneous physical and mental resemblances in MZ twins, and the possibility of this bias must be recognized.

Triplets and quadruplets show many peculiarities of twins in accentuated form. When these peculiarities are expected to complicate the interpretation of twin data, the higher order births should ideally be excluded or analyzed separately. Complete exclusion is not always possible. Persons who are known as twins to themselves and friends may be the survivors of a higher-order birth, and in pediatric problems, at least, this prenatal fact may be important. This is another reason for emphasis on birth records in twin studies.

Separate analysis of known triplets and quadruplets in a series of multiple births may be futile because of the small numbers. When such analysis reproduces a finding on twins in accentuated form it points to multiple birth as a distorting factor. Whether analyzed separately or not, triplet data are not amenable to all the twin methods. There may be no problem with two survivors, or with sets of mixed sex or zygosity, in which the odd member can be disregarded. All-identical or all-fraternal triplets provide three comparisons with two degrees of freedom while twins provide one comparison with one degree of freedom. Discordance in triplets yields two dissimilar pairs and one similar pair, while quantitative differences can be compared with twin differences only as estimates of correlation or variance (Richter and Geisser, 1960).

The most critical comparisons in a twin study are not always between identical and fraternal twins; in fact, when analysis is restricted to MZ-DZ comparisons valuable data are almost certain to be wasted. Comparison between concordant and discordant identical twins may reveal important clinical differences in a disease, or differences in genetic or environmental history. The normal partners of DZ twin patients may reveal signs of maternal effects, particularly when compared with siblings. Study of parents and siblings of twins offers the best prospect of obtaining Mendelian conclusions in the course of twin research (Kallman, 1959; Morton, 1962; cf. Gedda and Brenci, 1962), and enhances the value of metric observations also (Kempthorne and Osborne, 1961).

Although it goes far beyond the scope of twin research, the multiple abstract variance analysis method (Cattell, 1963) should be remembered in this connection because it is an extension of twin methods. Human behavior is generally not amenable to classical analysis of variance in

terms even of all known relevant variables. But in theory nearly the same objective can be attained by estimating components of variance in different but comparable series that permit solution of a set of simultaneous equations. This kind of study requires a magnitude and a sophistication of design at least comparable to a large, ideal twin study, and its only product is a collection of nature-nurture ratios that apply to a single and incompletely specified range of human environments. However, the testing of a large number of criteria in a single experiment may, as alleged, help to reveal the biologically most significant parameters and, by coincidence, even underlying single genetic factors (Fuller and Thompson, 1960; p. 343).

Miscellaneous Problems of Analysis

It has been stated repeatedly in the foregoing discussion that some types of error are unavoidable and must be allowed for. In addition, twin data present certain *avoidable* pitfalls in analysis and interpretation. One group of problems surrounds the practice of expressing twin data in terms of heritability. Another source of confusion or outright error is the statistical problem of ascertainment with reference to qualitative traits. Finally, the significance of differences between twins is often misconstrued.

In experimental and theoretical genetics, heritability is defined, for one usage, as that proportion of the total variance in a trait that is due to genotypic variance. The closest approach to this in twin data is the proportion of variance between DZ partners (V_{DZ}) that disappears when genotype is held constant (Newman, Freeman and Holzinger, 1937):

$$H = \frac{V_{DZ} - V_{MZ}}{V_{DZ}}$$

This formulation of heritability is rather unsatisfactory. Genotypic variance between DZ twins with respect to additive hereditary effects is theoretically only about half the total genotypic variance in the population (Kempthorne and Osborne, 1961). It is well that in the case of highly heritable traits the effect of this on the denominator is almost as large as that on the numerator. Two greater objections are that between-family environmental variance is omitted from the analysis, and certain components of environmental variance are held constant in MZ twins along with the genotype (Kempthorne and Osborne, 1961).

A similar formula is sometimes used for qualitative traits in terms of proportion of concordant pairs:

$$H = \frac{C_{MZ} - C_{DZ}}{1 - C_{DZ}}$$

Since concordance usually represents a threshold or cut-off point in an inaccessible continuum of resemblance, this formula cannot be translated into the usual concept of heritability.

Actually, estimates of heritability from twins are not used like those of experimental genetics, but only for comparison between traits and between studies. Since its standard error is not usually given and is difficult to compute, H is not very useful even if it were genetically meaningful. The simplest and probably the best way to summarize metric observations is with the intrapair correlation coefficient for each type of twin (Kempthorne and Osborne, 1961; Gottesman, 1963). If a single expression is wanted to gauge the strength of evidence for genetic factors, the variance ratio (Osborne and DeGeorge, 1959, p. 60) is less misleading than "heritability," but it is equally subject to environmental effects. For qualitative traits, concordance rates of MZ and DZ twins are more informative than H, and the approximate standard error of the difference for use in a one-tailed test is easily computed from the numbers of observations.

The statistical problems of ascertainment, i.e., detection of persons affected by a disease or qualitative trait are in principle well understood, but they take special forms in twins. They may be illustrated with two sets of data from the literature.

In a careful study of congenital malformations, Stevenson, Worcester and Rice (1950) found 16 sets of twins among 677 malformed births, an incidence of one set of twins for every 42 deliveries. In the population studied, twins occurred once in every 83 births, and the authors concluded that malformed twins had occurred twice as often as expected. This report is still cited sometimes as evidence for the abnormality of twin gestations. The authors neglected the fact that in a twin birth there are twice as many infants at risk as in a single birth. Actually, the incidence of twins in this population was *two* for every 84 infants, so that one in 42 is the expected proportion in the malformed series. When the material is thus analyzed in terms of individuals, the data show that the 16 pairs of twins included 20 affected infants. This is only 22 per cent above expectation, and actually without statistical significance. While there may be some excess of malformation in twins, faulty analysis led in this instance to rather great exaggeration of the problem.

As a second example, 21-trisomy is reported to occur concordantly in nearly all instances of affected monozygotic twins, but discordantly in

most affected dizygotic sets. Smith (1955) noticed that the proportion of identical twin sets reported in the literature was only one-sixth of all twin reports, instead of 26 per cent as expected among twins with the maternal age distribution found in 21-trisomy. Although he later corrected himself (1960), he originally concluded that many of the discordant sets reported as DZ had been misdiagnosed, and that there might in fact be a substantial number of MZ sets discordant for 21-trisomy. His conclusion found support in an excess of same-sex pairs among the reported DZ sets. Further reports have failed to confirm the excess of same-sex pairs among discordant twins, and when account is taken of the distribution of affected cases within pairs, the excess of DZ twins disappears also. Smith found 40 cases in 20 MZ pairs and 112 cases in 109 reportedly DZ pairs. If the risk of 21-trisomy is assumed to be the same in monozygotic and dizygotic twin infants, affected MZ twins (individuals) should be .35 times as numerous as affected DZ twins or 26 per cent of all affected twins. The accuracy of his maternal age analysis is apparently attested by the twin reports: the ratio of MZ to DZ individuals was .36; 26.3 per cent of the cases were members of monozygotic pairs.

These anomalies of twin data have been nicely explained by Stern (1958), who derived formulas for the expected frequency of twin pairs in a sample and for the expected proportions of the two types of twin. More general formulas were provided by Steinberg (1962), but the approach of both authors requires assumptions that are usually untenable for characters with incomplete penetrance, and the formulas are inapplicable to conditions of unknown and complex etiology as in the first example above. All the formulas assume, for example, that some individuals are genetically "unaffectable," and that all other individuals are uniformly affectable. In general the diathesis even for "constitutional" diseases is relative and may be present to some extent in all human genotypes. If a major gene is necessary but not sufficient to determine a trait, carriers of the gene may be presumed to vary widely in their tendency to manifest it. Another formula, suggested by Smith (1960), applies only when MZ twin are always concordant.

A much simpler and apparently more accurate approach to the evaluation of qualitative data in twin samples is to analyze them in terms of individuals (Allen, 1955). As shown in Table 4 and in the above two examples, this device eliminates the mathematical complexities of pairwise analysis. The main application is in estimating concordance. Under any analysis of a whole population, ascertainment anomalies do not occur with respect to concordance rate, which may be obtained directly as

the proportion of concordant pairs among all pairs. To estimate this population parameter from sample data a single formula is necessary, regardless of genetic mechanisms and regardless of the completeness of ascertainment:

$$c = \frac{C}{C + 2D}$$

where c is the estimated concordance rate in the population, C the number of index cases from affected concordant pairs and D the number of index cases from discordant pairs. When comparison is made between MZ and DZ twins or between categories within a type, the formula is applied to each body of data separately. The definition of an index case is important; it should exclude any subject who was investigated only because his partner was first found to have the condition, but it should not require "independent ascertainment" in the sense that the two partners must be found at different times or places. Such a requirement as the last would result in systematic exclusion of one twin of concordant pairs that stayed close together (*viz.*, MZ pairs) and double counting of concordant pairs that behaved independently (especially DZ pairs). Under incomplete ascertainment, concordance rates are exaggerated if concordant and discordant pairs are all counted once, a method commonly regarded as the most conservative.

The above concept of concordance is not applicable to normal qualitative traits. Here two or more concordant types will come into the sample. The statistical treatment of such data has been recently clarified by Gittelsohn and Milham (1964).

Frequently entangled with the calculation of twin concordance rates is the attempt to estimate penetrance of a gene or genotype; innumerable papers have been written on this question. In one example, not published, it was suggested that heterozygous or intermediate genotypes should be recognizable in identical twin pairs by their being discordant for the condition. Most authors have recognized such intermediate genotypes would produce pairs with two, one or none affected, according to chance. Few if any authors have recognized that this chance distribution may be modified by graded penetrance in different genotypes and by the inevitable correlation of all influences within pairs, to be expected as in litters of isogenic laboratory animals (Wright, 1934). If one of MZ twins manifests a trait or disease, the partner has at least all necessary genetic modifiers, and probably other genetic and environmental factors that favor manifestation. Thus virtually complete concordance in identical twins is, in theory, consistent with low penetrance of a major responsible gene in the population.

Estimation of penetrance from monozygotic twins may be harmless if it is used only for comparison with other twin data, but a simple concordance rate is as good for this purpose. Gross error is sure to result if the penetrance estimate is applied to the population, and if segregation ratios and gene frequencies are calculated on this basis. Concordance in MZ twins gives only the upper limit of penetrance. If diabetes is due to a single Mendelian factor, the observed MZ concordance rate of around 60 per cent (Harvald and Hauge, 1963) tells us that the responsible gene has a penetrance near to, or below,

$$\frac{2 \times .60}{1 + .60} = .75.$$

The correct figure might be 75 per cent or 20 per cent, as far as the twin data are concerned.

Like penetrance, intrapair variation in such dimensions as severity and age of onset in identical twins has little relevance to phenomena observed in the general population. Conversely, population data cannot safely be used to correct twin observations. For example, schizophrenia is usually considered to vary in age of onset from 15 to 45 years. But when one of identical twins contracts the disease at the earliest age it is not safe to assume that his partner's risk continues to age 45; he may be out of danger by age 20. In a series of discordant pairs some will later become concordant, but the proportion has to be estimated from historical or longitudinal data on identical twins. In principle, the same admonition applies to the study of dizygotic twins, or individuals with any degree of relationship. Follow-up studies or valid age-correction of cross-sectional data can be expected in general to decrease the apparent importance of genetic effects. If incidence is highest in the closet relatives of patients, their period of risk is also shortest, so that with passage of time the proportionate increase of cases among them should be smaller than among unrelated controls.

One further point is worth remembering about differences between MZ twins. Barring somatic mutation, observed differences are by convention attributed to "environment," but this is misleading. In a uniform environment genetically identical individuals can still be dissimilar, at least theoretically. Some events responsible for discordance, as for bilateral asymmetry, may be very subtle, for example statistical variation in a molecular process that is not controlled by either heredity or environment (Verschuer, 1954, p. 153). In such instances the most complete specification of environmental *differences* in discordant pairs could be irrelevant, and the observed concordance rate would provide no clue to

the mechanism. Equally inscrutable may be cases of heredity-environment interaction in ontogeny, leading practically to genetic differences within one genotype: once MZ twins undergo differentiation even from trivial environmental factors, their growth or behavior may come under control of different genes, present in both but effective in only one. At the present state of knowledge in human genetics there may be many such stubborn phenomena, rendering nearly valueless much twin research aimed at description or at estimating "heritability." Clearly, also, historical analysis of individual development is relevant for any but the simplest deductions from twin data.

Prospects

Despite the many difficulties of twin research and the present success of other methods, the future contribution of this specialty may be large. No material but twins can provide such convincing evidence for environmental etiologic factors prior to demonstration of the factors individually. The current nationwide study of twins in Denmark (Harvald and Hauge, 1963) is providing evidence of a large role of the environment in most of the diseases formerly regarded as "constitutional." Twin data can also suggest or confirm a particular etiology. Thus, in the case of 21-trisomy, twin data published by Orel, together with the nature of the disorder, apparently prompted Waardenburg's early correct suggestion of a chromosomal etiology.

A schema of appropriate qualitative interpretations of twin data, certainly not all-inclusive, is given in Table 5. Large and accurate studies are warranted if they can yield data on a number of pathologies simultaneously, because the relative *frequency* of a disease in twins is one of the primary requirements for further twin study. Concordance rates are not so critical. Very high or very low rates in either type of twins, or very similar rates in both types, have rather specific implications, and a moderate concordance rate for a supposedly genetic condition eliminates a complete genetic explanation. More precise determination of a concordance rate is generally not a worthwhile goal in itself. A detailed study of discordant MZ twins may support a prior hypothesis or suggest new ones, and this should be followed up by comparison with concordant or normal MZ twins or other material that offers appropriate control. Quantitative studies of MZ resemblances will have more relevance to important questions when the findings are focused on specific environmental influences, at least to exclude effects of the latter (Kempthorne and Osborne, 1961). The usefulness of twins in identifying biologically meaningful variables is a common but unsubstantiated assertion.

TABLE 5—*Possible Statistical Findings in a Twin Study of an Infrequent Qualitative Trait and Causes Implicated*

Concordance is regarded as low if less than 10 per cent, high if greater than 50 per cent, and complete if greater than 95 per cent

Condition Found to Have:	Possible Nongenetic Factors	Possible Genetic Factors
1. Higher frequency in all twins than in single-born	Prenatal, natal sociocultural	None implicated
2. Frequency elevated only in MZ twins	Prenatal, sociocultural	None implicated
3. Normal frequency, high concordance in all twins	Prenatal, sociocultural	None implicated
4. Normal frequency, high concordance in MZ twins	Sociocultural	Quantitative
5. Normal frequency, complete concordance in MZ, low concordonce in DZ twins	Cytoplasmic	Multiple (epistatic), new mutation, chromosomal aberration
6. Normal frequency, low concordance in all twins	Chance phenomena: development, physical environment	Heredity excluded

No matter how far human biology may advance, there will probably always be conditions or traits whose dependence on environment can profitably be explored with twins. Twin biology itself may help to elucidate problems in areas like reproductive physiology. The recent observations of plural births following use of drugs to correct infertility may provide clues to ovarian function (Riley and Evans, 1964), as may also the mathematical analysis of maternal age and parity in twinning. Study of twin placentas, membranes and immunological relations may continue to answer, as well as to raise, questions about early development. If there are still undiscovered teratogens like thalidomide or maternal rubella, attention may be called to them by birth defects occurring concordantly in DZ twins (Wildervanck, 1963).

The close similarity of the environment within most pairs of monozygotic twins, while sometimes a drawback, makes them ideally suited for testing the efficacy of medical or educational procedures. Ordinarily, large series of single individuals will suffice, but each pair of experimental observations on identical twins is worth about twenty in single individuals (Comstock, 1954). When observations are expensive or when individual variations may obscure the results, investigators should

consider the advantages of twin controls and should be prepared to use them. In a related application, identical twins may greatly aid the study of sequellae of injury or disease when concordant pairs are known (Dencker, 1959).

The greatest usefulness of twins may prove to be in developmental psychology and psychiatry. Behavior patterns are so variable and so complex even in infants that analysis into components and recognition of equivalent responses to different environments can probably proceed more rapidly with MZ twin pairs than with single subjects. Developmental psychology and twin research may both have to evolve a little further before this collaboration can be fruitful. For example, the value of twins in this and in many applications would be much enhanced by the availability of monozygotic pairs separated in early infancy (Shields, 1962). Adoption agencies routinely place twins in the same home, and deliberate separation would be looked upon as experimental. But bebecause twins are infrequent and abnormal by comparison with singleborn, the placing of twins in the same adoptive home is also experimental. In fact, it is rather difficult to find any aspect of the rearing of twins that is not by necessity experimental, and the best that can be done is to see that the experiments are well designed and well studied.

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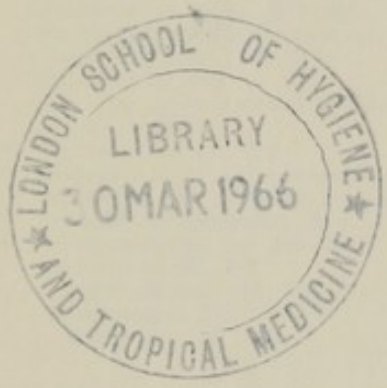
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