UNIT VII.
HEMOGLOBIN, SERUM GROUP SYSTEMS, HLA AND OTHER GENETIC MARKERS

SECTION 38. HEMOGLOBIN

38.1 Introduction

Hemoglobin (Hb) is the major protein of human red cells, comprising about 95% of their dry weight. Adult human blood normally contains from about 4 million to 6.5 million red cells per mm^3 blood, the average figure being slightly higher for men. Hemoglobin itself is present in concentrations of about 14 to 16 g per 100 $m\ell$ blood. It is the oxygen transporting protein in higher animals; without a molecule having its properties, complex multicellular aerobic life as we know it would not be possible.

Hemoglobin is one of the most extensively studied of all proteins, and its literature fills many volumes. As noted in section 5.1, it acquired its present name over 100 years ago (Hoppe-Seyler, 1864). In forensic serology, hemoglobin is important in two principal contexts: (1) Blood is normally identified in questioned samples by procedures designed to demonstrate the presence of hemoglobin; and (2) hemoglobin exhibits a very large number of genetic variants, a few of which are comparatively common and conveniently detectable by electrophoretic and/or isoelectrofocusing procedures. Many of the reactions of hemoglobin, which form the basis of blood identification techniques, depend upon the heme moiety of the molecule. These were discussed in detail in Unit II, sections 4 through 7. Hemoglobin variants, which can serve as genetic markers, are discussed in this section. Hb F, which was discussed in section 8.3.1 as a marker for the blood of fetuses and young children, is also discussed here. Studies on hemoglobin variants and their detailed structures have contributed significantly to our current understanding of molecular genetics.

38.2 Hemoglobin Structure

The structure of hemoglobin can be described at several different levels. First, the molecule is a subunit protein, and may be described in terms of the polypeptide chains that make it up. Second, the detailed structure of each type of subunit polypeptide chain can be described in terms of amino acid sequence, and in terms of helical and nonhelical regions. Finally, the x-ray data have made possible a detailed description of the three-dimensional structure for some of the hemoglobins. Detailed structures will be discussed briefly in the sections below.

The hemoglobin molecule is tetrameric, consisting of four associated polypeptide chains held together by noncovalent forces. One heme group is associated with each polypeptide chain. Most hemoglobins consist of two α chains and two non-α chains. The non-α chains can be β, γ, δ, ϵ or ζ. Normal adult hemoglobin is designated Hb A, and consists of two α and two β chains. Its structure may thus be written: $\alpha_2\beta_2$. Similarly, Hb A_2, a minor adult hemoglobin, has the structure $\alpha_2\delta_2$. Normal fetal hemoglobin, Hb F, is $\alpha_2\gamma_2$, and so forth. Hemoglobins exist which contain only one kind of chain: Hb H is β_4, for example, and Hb Bart's is γ_4.

Jones (1961) pointed out that hemoglobin structural heterogeneity can be classified as follows: (1) Maturation heterogeneity, which refers to the fact that different hemoglobins are normally synthesized during different stages of development. There are embryonic, fetal and adult hemoglobins. (2) Minor hemoglobin heterogeneity, which refers to the presence of small amounts of structurally different but normal hemoglobins along with the major component characteristic of a particular stage of development; and (3) genetic heterogeneity, which refers to the various "abnormal" hemoglobin variants. Many of these are thought to be the result of point mutations, and with a few exceptions, variant hemoglobins are very rare. The excellent review of hemoglobins by Huisman (1969) was organized according to this classification of heterogeneity.

38.2.1 Normal adult hemoglobins

The structural studies on normal adult hemoglobins were prompted in part by the recognition that the sickle cell condition represented a discrete molecular alteration in the Hb molecule. Structural studies on normal adult and fetal, and variant hemoglobins were carried out simultaneously in an effort to relate the detailed structures to the genetics. Most of these studies occurred in conjunction with the development of present-day understandings of biochemical genetics (section 1.2.2), and have contributed importantly to them.

The major normal adult hemoglobin is called Hb A. It has been called Hb A_1, A_0 and A_{II} at different times (Holmquist and Schroeder, 1966a), but these latter usages are now discouraged (see in section 38.2.3.6). Hb A is a tetramer composed of two α and two β chains, and its structure is written $\alpha_2\beta_2$. Each polypeptide chain is associated with a heme group (Figures 4.4 and 4.5). Intact Hb A thus has 4 heme groups, and its MW is 64,450. The complete amino acid sequences of the α and β chains were worked out in the early 1960's, and are shown in Figure 38.1 (Braunitzer *et al.*, 1961; Konigsberg *et al.*, 1961). The sequencing studies have been well reviewed by Braunitzer *et al.* (1964). Part of the stability of protein molecules arises from the α-helical arrangement of the polypeptide chains (section 1.1.2.1). This structural feature was predicted by Pauling and Corey, and has since been found to occur widely in nature (Pauling, 1960). The helical structure of the polypeptide chains of Hb has been worked out using x-ray crystallographic and other techniques. The polypeptide chains consist of a series of helical regions which are periodically interrupted. These helical regions are indicated by upper case letters, as shown

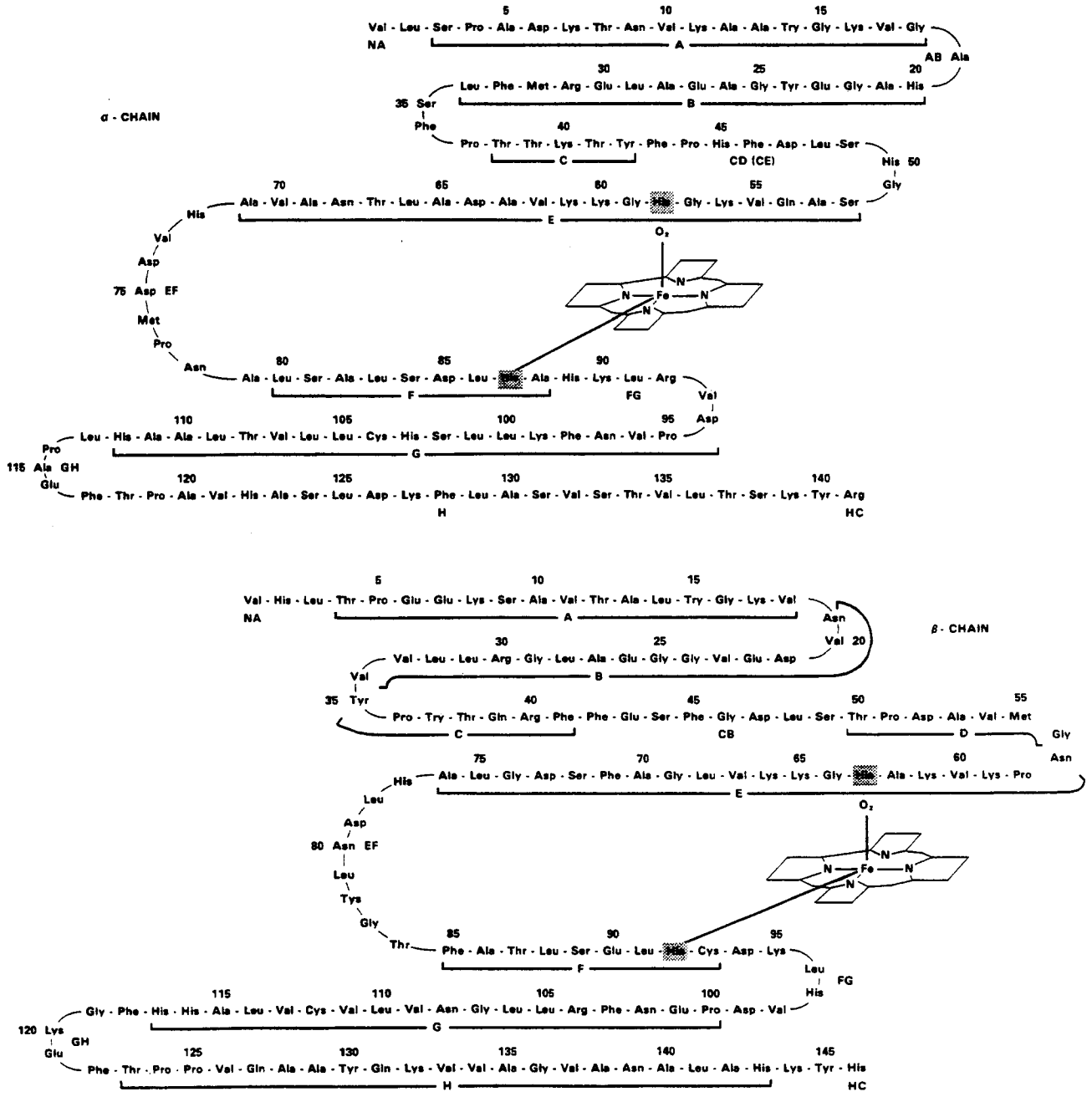


Figure 38.1 **α and β Chain Sequences in Hemoglobin.**

Helical regions are denoted by capital letters. The α 50 - 51 residues are denoted D6 - D7, D1 - D2, and CD8 - CD9 by different authors. There are no non-helical sequences BC and DE in α; since there is no helical sequence D, CD is followed by E. There are no nonhelical sequences AB, BC and DE in β. The proximal and distal histidines, which interact with heme, are boxed.

in Figure 38.1. The location of a particular amino acid residue in the chain may thus be indicated in two ways: first, all the amino acid residues are numbered sequentially from the N-terminal end of the chain (as is the usual convention); and second, the position of the amino acid in a particular helical region (numbering from N-terminal to C-terminal) may also be given. Transitional regions, which lie between two helical regions, are designated using two letters, the one representing the immediately preceding (N-terminal side) helical region, and the one representing the immediately following (C-terminal side) one. The N-terminal amino acids which precede the first (A) helical region are denoted "NA", while the C-terminal amino acids which follow the last (H) helical region are denoted "HC". A few examples of this nomenclature (see in Figure 38.1): (1) the N-terminal amino acid of the α chain, valine, can be called "1", or "NA 1"; (2) the Asn residue of the α chain is "68" or "E17"; (3) the 6th residue of the β chain is Glu, and is designated β6(A3); and (4) the Phe residue of the β chain at position 42 is β42(CD1). The helical designations are extremely useful in comparing different chains for structural homologies and differences. Present nomenclature recommendations call for the use of both the absolute position and the helical region position designations in specifying a particular residue (see in section 38.2.3.6). The exact location of the helical regions in the polypeptide, and the exact three-dimensional structure of the hemoglobin molecule have been determined for the most part by the x-ray crystallographic studies of Perutz, Kendrew and their collaborators in England. These studies actually began in the 1940's, but were interrupted by World War II. They were continued after the war, and were directed at determining the complete structures of human and animal hemoglobins and myoglobins. The solution of these structures represents one of the brightest moments in protein chemistry. The structure of horse Hb was obtained before that of the human molecule (Perutz, 1962 and 1965; Perutz *et al.*, 1960, 1964 and 1965; Muirhead and Perutz, 1963). In these papers as well as in many of the reviews cited in this section will be found drawings of the three-dimensional conformation of hemoglobin and photographs of the molecular models that were constructed based upon the measurements.

It has been known for many years that Hb A is not the only hemoglobin in adult red cells. Hb A_2 was observed by Kunkel and Wallenius in 1955. Chromatographic techniques have revealed the presence of several "minor" hemoglobins in adult blood. Huisman *et al.* (1958) detected two minor fractions in addition to A. Others detected a number of minor components, which were designated A_I, A_{III}, A_1, A_2, A_3 and so forth. Subfractions related to A_I were called A_{Ia} through A_{Ie} (Allen *et al.*, 1958; Clegg and Schroeder, 1959; Schnek and Schroeder, 1961; Jones and Schroeder, 1963; Atassi, 1964; Holmquist and Schroeder, 1964, 1966a and 1966b). The nomenclature of the components is quite complex, because different workers have used different designations, and because the number of minor hemoglobins seen depends on the separation technique employed. Hb A_2 is genetically distinct from Hb A. In 1961, Ingram and Stratton discovered that Hb A_2 is not made up of the same polypeptide chains as Hb A, consisting instead of α chains and δ chains. Hb A_2 has the subunit formula $\alpha_2\delta_2$. The sequence of the δ chain has been determined, and is shown in Figure 38.2. Hb A_2 represents about 2% of hemoglobins as a rule, but this proportion can be altered by pathological conditions. The other "minor" hemoglobins are not as well characterized in general, but are thought to result from post-synthetic alterations of the polypeptide chains, rather than from distinct genetic loci. Hb A_{Ic}, for example, appears to have hexose (probably glucose) condensed with the N-terminal amino acid of the β-chain (Holmquist and Schroeder, 1964, 1966a and 1966b; Lehman and Huntsman, 1974). Hb A_{Ic} comprises 5 to 7% of normal hemoglobins. Another minor hemoglobin may come about through the combination of Hb A with glutathione. It is now recommended that the minor hemoglobins be designated Hb A_2 ($\alpha_2\delta_2$) and Hb A_I (fast moving zone in electrophoresis at alkaline pH). Components of Hb A_I are Hb A_{Ia}, A_{Ib}, etc., and components of the subfractions are A_{Ia1}, A_{Ia2}, etc.

38.2.2 Normal embryonic and fetal hemoglobins

In 1867, Körber first described the differentiation of animal hemoglobins by the rate at which they denature in alkali. In addition, he found that fetal Hb was much more resistant to alkali denaturation than adult Hb. This property may still be used to distinguish between fetal and adult hemoglobins (section 8.3.1). The principal hemoglobin of cord blood at birth is called Hb F, normal fetal hemoglobin. Hb F is found in fetuses after about 10 weeks of gestation. Its level increases for a time until, at birth, it is found together with Hb A. After about 6 months, Hb F all but disappears. Hb F comprises only about 1% of normal adult blood hemoglobins (Chernoff, 1953b). Hb F can be distinguished from Hb A by differential sensitivity to alkali denaturation (Körber, 1867; Singer *et al.*, 1951), chromatographic techniques (Allen *et al.*, 1958; Huisman *et al.*, 1958), electrophoresis (Culliford, 1964; Huehns, 1968) or by immunological methods (Chernoff, 1953a and 1953b; Goodman and Campbell, 1953; Diacono and Castay, 1955). Hb F is made up of two α chains and two γ chains (Schroeder and Matsuda, 1958). Its tetrameric formula is $\alpha_2\gamma_2$. The α chains in Hb A and Hb F are identical (Schroeder *et al.*, 1963a). The amino acid sequence of the γ chain has been worked out (Schroeder *et al.*, 1963b) and is shown in Figure 38.2. It is now known that at least two slightly different γ chains are normally synthesized, and occur in Hb F (see below). There are minor components of Hb F, just as there are of Hb A (Allen *et al.*, 1958). Hb F_I, which can account for 10% of the fetal Hb, differs from Hb F in that the N-terminal residue of the γ chain is acetylated (Schroeder *et al.*, 1962). It was first thought that Hb F_I was $\alpha_2\gamma^F\gamma^N$, where γ^F was the usual Hb F γ chain, and γ^N was the N-acetyl-Gly. . . . . γ chain. It appeared that there was more to it, however (Huehns and Shooter, 1966), and with the finding of the different kinds of γ chains, the detailed

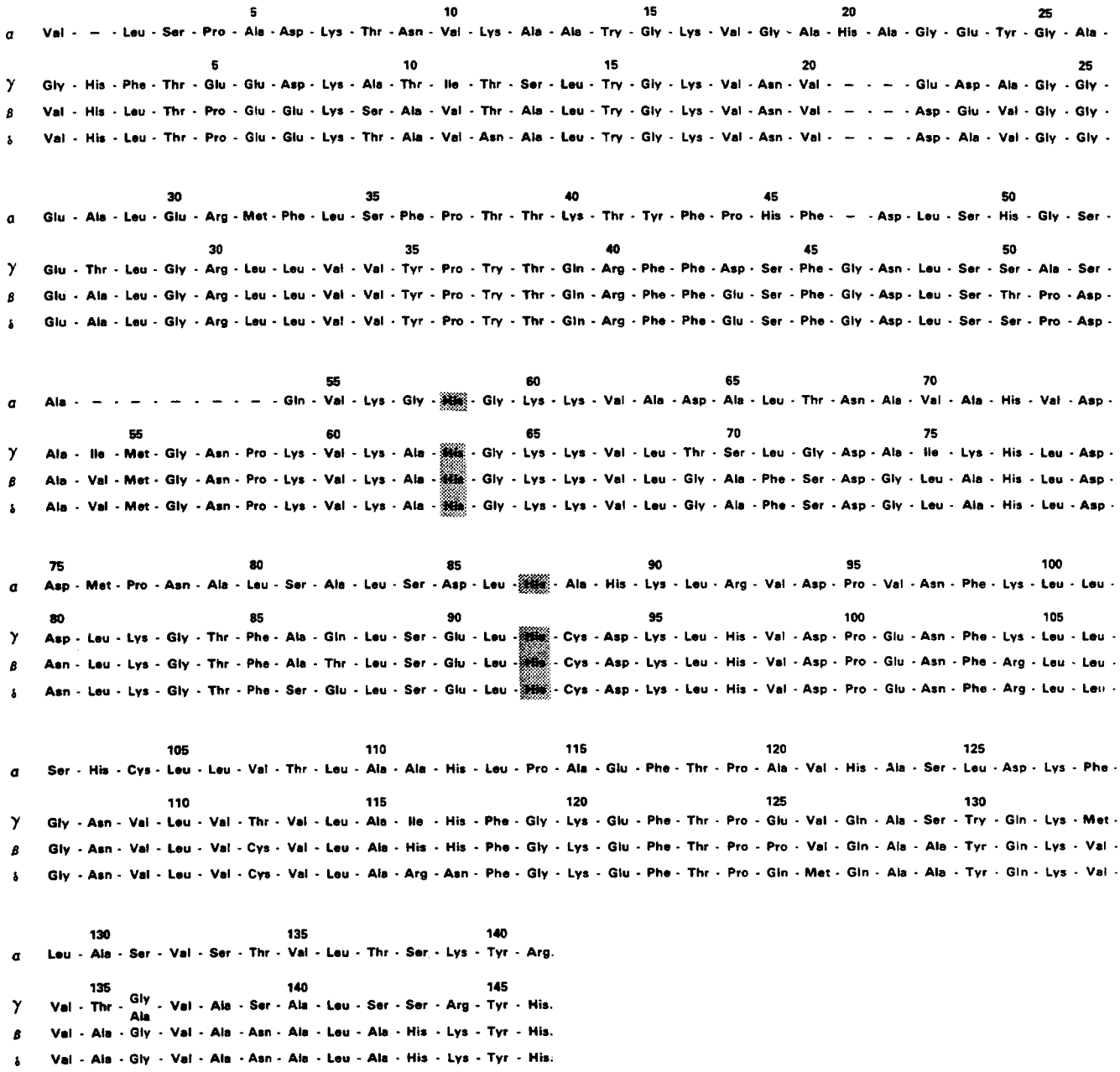


Figure 38.2 Sequences of the α, β, γ and δ Chains of Hemoglobin.

Sequences are arranged to show the maximum sequence homologies between chains. Large dashes represent 'Braunitzer gaps' (see text). The histidyl residues which interact with heme are half-toned. Not all authors place the gaps identically.

structure of Hb F_1 will undoubtedly be found to be more complex than originally thought.

The sequences of the α,β,γ and δ chains are shown together in Figure 38.2. One of the interesting features of the sequences, in view of what is now known about the genetics of Hb, is the similarity of sequence structure among the different chains. This similarity is called structural or chemical "homology". The structural homology can be maximized by introducing theoretical "gaps" into the chains at certain points. These are called "Braunitzer gaps", and they do not really exist, of course. Figure 38.2 has the Braunitzer gaps in order to illustrate the maximum chain homologies.

In 1954, Drescher and Künzer noticed that there seemed to be another hemoglobin in fetal blood that differed from Hb F. Its alkali denaturation curve was intermediate between that of Hb A and Hb F. Halbrecht and Klibanski (1956) confirmed another hemoglobin in very early fetuses by electrophoretic and alkali denaturation differences. Allison (1955) referred to this early hemoglobin as "primitive". In 1961, Huehns *et al.* found not one but two hemoglobins in early fetuses (less than 10 weeks of gestational age), which they called Hb Gower 1 and Hb Gower 2. These names are still used. Additional studies on these "embryonic" (as against "fetal") hemoglobins indicated that they contained a new polypeptide chain, designated ϵ. Hb Gower 2 had the structure $\alpha_2\epsilon_2$, while Gower 1 was thought to consist entirely of the new chain, i.e. ϵ_4 (Huehns *et al.*, 1964a and 1964b). In very young embryos, Hb Gower 1 is the dominant Hb (Hecht *et al.*, 1966), and as the embryo develops the proportion of Gower hemoglobins decreases as that of Hb F increases. In 1967, Capp *et al.* found a new hemoglobin in a baby girl in Portland, Oregon, who had been born with multiple abnormalities. This hemoglobin had two γ chains and two chains that were not yet identified, and was called Hb Portland 1. This Hb was soon found in normal infants as well (Hecht *et al.*, 1967), and is now believed to be a normal embryonic hemoglobin. The second kind of polypeptide chain in Hb Portland 1 was a new one, and was designated ζ (Capp *et al.*, 1970). Hb Portland 1 is thus $\gamma_2\zeta_2$, and it is now thought that Hb Gower 1 is $\zeta_2\epsilon_2$, instead of ϵ_4 as first thought. There are thus three embryonic hemoglobins, distinct from Hb F.

As noted briefly above, there is some further complexity in Hb F, which has the structure $\alpha_2\gamma_2$. Additional sequence studies on the γ chains revealed a rather startling finding: that Hb F normally contains two different kinds of γ chains (Schroeder *et al.*, 1968). These chains differ only at position 136, which may be occupied by Gly or by Ala. The resulting γ chains are designated $^{G}\gamma$ and $^{A}\gamma$, respectively. All the Hb F samples studied had both kinds of chains, from which had to be drawn the extraordinary genetic conclusion that there were at least two γ chain loci, and that they were nonallelic. There is now good evidence for the nonallelic γ chain loci, and the matter is discussed further in section 38.3. There is another complexity in γ chain structure. Ricco *et al.* (1976) found a Hb F with still a different γ chain. The new chain had Thr at position 75(E19) instead of Ileu, and was designated $^{T}\gamma$. Schroeder *et al.* (1979) believe that $^{T}\gamma$ is a product of a mutant $^{A}\gamma$ locus, and suggested that the chain be called $^{TA}\gamma$. A review of fetal and embryonic hemoglobins was given by Lorkin (1973). Recommendations have recently been made by an international body regarding standardized Hb F preparations (International Committee, 1979).

The tetrameric structural formulas of the normal adult, fetal and embryonic hemoglobins are summarized in Figure 38.3.

Adult

Hb A	$\alpha_2\beta_2$
Hb A_2	$\alpha_2\delta_2$

Fetal and Embryonic

Hb F	$\alpha_2\gamma_2$ ($\alpha_2{}^{G}\gamma{}^{A}\gamma$)
Hb Gower I	$\zeta_2\varepsilon_2$
Hb Gower II	$\alpha_2\varepsilon_2$
Hb Portland I	$\gamma_2\zeta_2$

Figure 38.3 Tetrameric Structure of Adult, Fetal and Embryonic Hemoglobins

38.2.3 Genetic variants of hemoglobin

38.2.3.1 Introduction. Obviously, the "normal" hemoglobins just discussed are genetically different, in that different genes are responsible for the synthesis of the different chains comprising them. The "genetic variant" hemoglobins, however, are those which exhibit structural differences from the "normal" hemoglobins. Hundreds of such variants are now known, and in most cases the exact structural difference is known. Many of the variants are very rare and can be explained by mutations. A few variant kinds of Hb reach polymorphic frequencies in certain populations, presumably because there is some selective advantage to their possessors. In some cases, the selective advantage can be explained, such as in the case of Hb S.

The first "abnormal" hemoglobin to be studied in detail was Hb S, or sickle cell hemoglobin. The recognition that sickle cell trait and sickle cell anemia represented the heterozygous and homozygous conditions of a variant gene, and the finding that Hb S differed from Hb A in a single amino acid, ushered in an era in human biochemical genetics. Hemoglobin thus became a model system for relating genetic variation to protein structure at the molecular level. Developments in protein separation methodology along with the solutions to the three-dimensional structure of hemoglobin have made it possible to find many variant hemoglobins, and to diagnose the differences in structure between normal and variant polypeptide chains. Since Hb A and Hb F are constructed from α, β and different γ chains, each coded for by different genetic loci, hemoglobin

variants are often categorized according to the chain in which the variation occurs, i.e. α chain variants, β chain variants and γ chain variants. The simplest type of Hb variants have a single amino acid substitution in one of the chains. There are variants which have two substitutions in the same chain. There are also more complex variants, such as those with extended chains, frameshift mutations, deleted residue chains and the so-called fusion hemoglobins.

38.2.3.2 α Chain variants. The α chain consists of 141 amino acids, and single amino acid substitution variants have now been found at many of the positions. Huisman (1969) noted that 40 possible variants had been reported, 22 of which had been fully characterized. Lehmann and Huntsman (1974) could list about 65 α chain variants in which the substitution had been characterized. In 1975, the International Hemoglobin Reference Center was established at the Medical College of Georgia in Augusta. Its purpose is to consolidate and organize all the information about Hb variants that appears in the literature, and which may be sent to the Center. A number of hemoglobin variants have several names, because some variants discovered in different laboratories and differently named have later turned out to be the same. The Center periodically communicates lists of variants in the new journal *Hemoglobin*. These lists are cited in this book as "IHIC Variant List, Year published", and the references are given under the same designation in the reference lists. By late 1976, the number of defined α chain variants was 73 (IHIC Variant List, 1976a), and is probably greater now. A few α chain variants (not selected for any particular reason) are shown in Table 38.1.

38.2.3.3 β Chain variants. The first variants of hemoglobin described were β chain substitution variants, and a description of the way the early variants were characterized is almost equivalent to a description of the early development of hemoglobin biochemical genetics. Not surprisingly, the first variants studied were those that are comparatively common. Because of their comparatively more frequent occurrence in populations, they are also the most important genetic markers from a forensic point of view.

Sickle cell hemoglobin is the "premiere" variant of Hb A. Many of the pioneering studies on Hb have been carried out on sickle hemoglobin (Hb S). The sickling of red cells from a patient was first noted by Dr. Herrick in Chicago, in 1910. Hahn and Gillespie (1927) studied the sickling behavior of red cells from affected people, and showed that the red cells could be induced to sickle *in vitro* under conditions of low oxygen concentrations, and that they would regain their shape if the O_2 level was brought back to normal. Detailed studies on the medical and physiological consequences of sickle cell trait and anemia have been carried out over the years by Diggs (Diggs and Ching, 1934; Diggs, 1956 and 1965), among others. The condition was recognized as being inherited, and largely restricted to Black populations, in 1934. Before the genetic details of sickle cell had been worked out, a government hospital worker in Rhodesia looked at the frequencies of "sickle" in two different population groups, and concluded that this characteristic could serve as a means of differentiating them anthropologically (Beet, 1946). In addition, he recognized that "sickle" was inherited in a simple Mendelian fashion, based upon studies of a large pedigree (Beet, 1949).

In 1949, Neel proposed a formal genetic scheme for the inheritance of sickle cell trait and disease. He regarded the sickle gene as a dominant characteristic. Very shortly thereafter, Pauling and his collaborators showed that Hb A and Hb S could be separated electrophoretically, and further that sickle cell trait people had about half Hb A and half Hb S, while sickle cell anemics had all Hb S. This finding indicated a simple codominant manner of inheritance in which both genes were expressed in heterozygotes. The presence of an S gene could thus be shown to produce a separate molecule, and Pauling *et al.* (1949) termed sickle cell anemia a "molecular disease". This new concept represented a different way of looking at numerous metabolic disease states, and hemoglobin served as the model system in its subsequent development (Itano, 1953). Sickle cell Hb was clearly different from normal adult Hb (Pauling *et al.*, 1949; Allison, 1957). In 1956, Ingram and his collaborators began publishing their studies on the exact chemical difference between Hb A and Hb S, the results of which opened up the present period of molecular genetic investigation. By a technique called peptide mapping, Ingram (1956) showed that Hb A and Hb S differed in a single peptide. Subsequent studies quickly demonstrated that Hb S differed from Hb A in a single amino acid residue (Ingram, 1957, 1958 and 1959; Hunt and Ingram, 1958a and 1958b). Hb S contained Val where Hb A had Glu. Hemoglobin C (Itano and Neel, 1950) was soon found to have a Glu→Lys substitution at the same position as the one in Hb S (Hunt and Ingram, 1960). Hemoglobin D was first described in Los Angeles, but reaches high frequencies in the Punjab. It is thus called Hb D Los Angeles, Hb D Punjab, and a variety of other names; it is often simply called Hb D, although there are other hemoglobins called "D", which are different. The comparatively common Hb D (Los Angeles or Punjab) has a Glu→Gln substitution at position 121 (Baglioni, 1962a). Hb D Ibadan, for example, is a β87 Thr→Lys variant, while Hb D Iran is β22 Glu→Gln. Hb E was found by Itano *et al.* (1954) using electrophoretic separations. It was shown to represent a Glu→Lys substitution (Hunt and Ingram, 1959 and 1961).

There are now known to be many β chain variants, and a sample of them is shown in Table 38.1. Huisman (1969) listed 45 variants. Lehmann and Huntsman (1974) showed over 120. The IHIC Variant Lists (1976b, 1977a and 1978) showed 138, 164 and 173 variants, respectively.

There are some hemoglobins that have a double substitution in the β chain. Hb C Harlem is a good example (Bookchin *et al.*, 1967). Its β chains are substituted 6Glu→Val and 73Asp→Asn. Moo-Penn *et al.* (1975) found a quite remarkable 35 year old Black man who was heterozygous for Hb S and Hb C Harlem.

38.2.3.4 γ Chain variants. There are fewer γ chain substitution mutants characterized than there are α or β ones.

Table 38.1 Some Hemoglobin Variants

Common Name	Synonyms	Scientific Designation	Reference
α Chain			
J Toronto		α 5 (A3) Ala → Asp	Crookston et al., 1965
Anantharaj		α 11 (A9) Lys → Glu	Pootrakul et al., 1975
I Philadelphia	I; I Texas; I Burlington	α 16 (A14) Lys → Glu	Beale and Lehmann, 1965
Fort Worth		α 27 (B8) Glu → Gly	Schneider et al., 1971
L Ferrara	Umi; Kokura; Michigan I; Michigan II; Yukuhashi II; L Gaslini	α 47 (CD5 or CE5) Asp → Gly	Bianco et al., 1963
Arya		α 47 (CD5 or CE5) Asp → Asn	Rahbar et al., 1975
Montgomery		α 48 (CD6 or CE6) Leu → Arg	Brimhall et al., 1975
Mexico	J Mexico; J Paris II; Uppsala	α 54 (E3) Gln → Glu	Jones et al., 1968; Fessas et al., 1969
J Rajappen		α 90 (FG2) Lys → Thr	Hyde et al., 1971
Chiapas		α 114 (GH2) Pro → Arg	Jones et al., 1968
β Chain			
S		β 6 (A3) Glu → Val	Ingram, 1957 and 1959
C		β 6 (A3) Glu → Lys	Hunt and Ingram, 1960
Saki		β 14 (A11) Leu → Pro	Beuzard et al., 1975
J Baltimore	J Trinidad; J Ireland; J Georgia	β 16 (A13) Gly → Asp	DeJong and Went, 1968
G Taipei		β 22 (B4) Glu → Gly	Blackwell et al., 1969
E		β 26 (B8) Glu → Lys	Hunt and Ingram, 1961
Alabama		β 39 (C5) Gln → Lys	Brimhall et al., 1975
Austin		β 40 (C6) Arg → Ser	Moo-Penn et al., 1977
Athens, Georgia	Waco	β 40 (C6) Arg → Lys	Moo-Penn et al., 1977
G Copenhagen		β 47 (CD6) Asp → Asn	Sick et al., 1967
J Kaohsiung	J Honolulu	β 59 (E3) Lys → Thr	Blackwell et al., 1971
J Cambridge	J Rambam	β 69 (E13) Gly → Asp	Sick et al., 1967
Atlanta		β 75 (E19) Leu → Pro	Hubbard et al., 1975
D	D Los Angeles; D Punjab; D Chicago; D North Carolina; D Portugal; Oak Ridge	β 121 (GH4) Glu → Gln	Baglioni, 1962a; DeJong and Went, 1968
Beograd		β 121 (GH4) Glu → Val	Efremov et al., 1973
γ Chain			
F Auckland		^{G}γ 7 (A4) Asp → Asn	Carrell et al., 1974
F Kuala Lumpur		^{A}γ 22 (B4) Asp → Gly	Lie-Injo et al., 1973
F Victoria Jubilee		^{A}γ 80 (EF4) Asp → Tyr	Ahern et al., 1975
δ Chain			
A_2 Roosevelt		δ 20 (B2) Val → Glu	Rieder et al., 1976
A_2 Melbourne		δ 43 (CD2) Glu → Lys	Sharma et al., 1974
A_2 Indonesia		δ 69 (E13) Gly → Arg	Lie-Injo et al., 1971
A_2 Coburg		δ 116 (G18) Arg → His	Sharma et al., 1975
Deleted Residues			
Leiden		β 6 or 7 (A3 or A4) Glu missing	DeJong et al., 1968
Lyon		β 17-18 (A14-A15) Lys-Val missing	Cohen-Solal et al., 1974
Freiburg		β 23 (B5) Val missing	Jones et al., 1966
Extended Chains			
Constant Spring		α + 31C (142 Gln)	Clegg et al., 1971
Fusion Hemoglobins			
Lepore Hollandia		δ (1-22) β(50-146)	Barnabas and Muller, 1962
Lepore Baltimore		δ (1-50) β(86-146)	Ostertag and Smith, 1969

The finding that Hb F normally contains two different γ chains, $^{G}\gamma$ and $^{A}\gamma$ (section 38.2.2), means that the presence of either Gly or Ala at γ136 is normal. Variants at other positions thus have to be characterized at γ136 as well as at the "variant" position. Further, γ75 can be occupied by either Thr or by Ileu. It is now recommended (see in section 38.2.3.6) that these chains be separately designated: $^{A}\gamma^{I}$ = γ75Ile; 136Ala; $^{A}\gamma^{T}$ = γ75Thr; 136Ala; $^{G}\gamma^{I}$ = γ75Ile; 136Gly. There are now about 15 γ chain variants (IHIC Variant Lists, 1977b), and the subject has been recently reviewed by one of the pioneers in the field (Schroeder, 1977). A sample of γ chain variants is shown in Table 38.1.

38.2.3.5 Other variants and other hemoglobins. A number of variants of the δ chain have been found in Hb A_2. The IHIC Variant Lists (1977b) show 10, and a sample of them is included in Table 38.1. A few other types of variants are known. The majority of variants represent single amino acid substitutions; a few (like Hb C Harlem, mentioned in section 38.2.3.3) have a double substitution. Three other types of variants will be mentioned here: deletions, extended chains and fusion hemoglobins. Deletion variants are those which lack one or more amino acids found in the usual polypeptide chain. A few of them are shown in Table 38.1. Hb Freiburg is an example. It is missing the Val residue at β23, and is designated β23Val→0. The IHIC Variant Lists (1977b) show 10 deletion variants. Extended chain hemoglobins have more amino acid residues on the C-terminal end of the chain than are normally found. Hb Constant Spring (Clegg *et al.*, 1971), for example, has 172 residues in the α chain instead of 141. Some authors show it as "α141 31 additional residues" in α chain variant lists. There are 7 extended chain hemoglobins in the IHIC Variant Lists (1977b). The fusion hemoglobins are very interesting, and their existence has implications for the structure and arrangement of the β, γ and δ chain genes at the molecular level. In 1958, Gerald and Diamond found a most peculiar hemoglobin in Boston, which they called "Lepore". Characterization of the molecule (Baglioni, 1962b) showed that the non-α chains appeared to be made up of an N-terminal section of the δ chain attached to a C-terminal section of the β chain. The "break" occurs between what would be δ87 and β116, but because of the sequence homology of the chains (Figure 38.2), the exact location cannot be determined. This δ–β fusion chain was apparently made by a new gene having portions of δ and portions of β. There are other examples of these δ–β fusion mutants now (Table 38.1), and they are sometimes called "Lepore" hemoglobins. Individual examples are distinguished by their place of origin or discovery, such as Hb Lepore Boston, Hb Lepore Hollandia, etc. Huisman *et al.* (1972) found a hemoglobin, Hb Kenya, whose non-α chain is a γ–β fusion hybrid. The "break" is between γ81 and β86. The IHIC Variant Lists (1977b) show 7 fusion hemoglobins.

There are a few other hemoglobins which should probably not be considered "variants" strictly speaking, though they are abnormal. They are usually associated with anemias and other hematological disorders.

There is a class of anemia syndromes known as *thalassemias*. The term is derived from the ancient Greek word for "the Sea" (meaning the Mediterranean). It turns out to mean "sea in the blood", rather than Mediterranean anemia, which it was coined to denote (Lehmann and Huntsman, 1974). The usage has persisted in the literature, even though the thalassemia syndromes are not restricted to Mediterranean peoples. In effect, a thalassemia syndrome is characterized by an imbalance of α or β polypeptide chain production. α-Thalassemias are those in which there is underproduction of α chains, while β-thalassemias represent underproduction of β chains. β-Thalassemia is the classical kind of Mediterranean anemia (Cooley's anemia), and is the most important in terms of frequency of occurrence. It is controlled by a gene which behaves like an "allele" of the β chain structural gene. The terms "β thalassemia major" and "β thalassemia minor" indicate homozygosity and heterozygosity, respectively, for the gene, although clinicians may use these terms to indicate the severity of the clinical manifestations. In the α-thalassemias, there is an underproduction of α chains and a corresponding excess of β chains in adults and of γ chains in fetuses and children. Before any of this was clearly understood, two hemoglobins without α chains were described; Hb H is β_4 and Hb Bart's is γ_4. The latter was apparently named after St. Bartholomew's Hospital, where the baby in whom it was first seen was born (Lehmann and Huntsman, 1974). It is now known that Hb H and Hb Bart's arise by the same basic mechanism. There are two models for the inheritance of α-thalassemia. In the first, there are two allelic α-thalassemia genes: the severe classical α thalassemia 1 gene and an α thalassemia 2 gene. The four possible combinations give rise to a series of increasingly serious clinical conditions. The *αthal* 2 heterozygotes are least affected, while the *αthal* 1 homozygotes are stillborn in the last weeks of pregnancy. In the second model, one must accept that the α chain structural gene is duplicated (for which there is some evidence). Then, either one, two, three or all four α chain genes can be affected by thalassemia, again giving rise to the series of four conditions of increasing clinical severity (Kattamis and Lehmann, 1970). The full explanation for all of the thalassemia syndromes is somewhat more complex than has been indicated, and there is still more to be learned about them. Thalassemia syndromes have recently been reviewed by Bank (1978).

Methemoglobinemia simply means that met-Hb is present in the red cells at clinically significant levels. Met-Hb is hemoglobin in which the heme iron is in the Fe^{3+} state, and it cannot bind oxygen. Methemoglobinemia can come about in a number of ways, only one of which will be discussed here. As shown in Figure 38.1, the heme moiety of Hb is slung between two histidyl residues (His58 and His87 in the α chain, and His63 and His92 in the β). The histidyl-nitrogen of the proximal histidine (α87 and β92) is coordinated to the Fe^{2+} of heme, while the distal histidyl-N (α58 and β63) per-

mits space for the binding of molecular oxygen. This structure forces the iron to retain the ferrous state. A few hemoglobins have been found in which an amino acid substitution occurs at α58 or β63. These disrupt the stability of the heme-α chain or heme-β chain interaction, and allow methemoglobin to form in quantity. As a result, they are called the "M" hemoglobins. Substitutions at other positions in the chains can disrupt the heme-peptide chain stability too. Hb M Milwaukee (β98Val→Glu) causes the heme iron to take on the ferric state.

A final condition that will be mentioned is called "hereditary persistence of fetal hemoglobin" or HPFH. There are a number of anemias and other conditions that can lead to the presence of Hb F after infancy. In HPFH, however, there is continued production of Hb F into the post-infancy years because β and δ chain synthesis has not been "switched on". There are several possible genetic explanations for this phenomen, but it has been attributed to the presence of a "high F" gene. While this explanation is a convenient way of thinking about HPFH, it is probably too simple. Indeed, different examples of HPFH may have different explanations. The "high F" heterozygotes have 10% to 40% Hb F. Homozygotes who have been studied make no Hb A nor any A_2.

The material in section 38.2 has been reviewed on many occasions. The older literature is well reviewed by Braunitzer *et al.* (1964), Schroeder and Jones (1965) and by Huehns and Shooter (1965). More recently, there is Giblett (1969), Huisman (1969), Lehmann and Huntsman (1974), Bunn *et al.* (1977a) and Bank *et al.* (1980). The papers in the major N. Y. Academy of Sciences conference (Kitchin and Boyer, 1974) covered many aspects of hemoglobin. The so-called hemoglobinopathies are well reviewed by Bunn *et al.* (1977b) and by Huisman and Jonxis (1977). The latter book gives detailed procedures for the characterization of hemoglobin variants.

38.2.3.6 Hemoglobin nomenclature. Just as in many other genetic marker systems, the nomenclature of Hb variants has developed somewhat unsystematically. More recently, there have been efforts to systematize it. Some old names are so entrenched in the literature that they have been retained. With the IHIC now established (section 38.2.3.2), nomenclature standardization should prove to be considerably easier than previously.

In the early years of Hb variant research, capital letters were used to designate each new kind of hemoglobin that was found. Hb A was normal adult hemoglobin. The letter "B" was apparently skipped because sickle cell Hb had sometimes been called B. Everyone agreed on Hb S for sickle cell Hb, and soon after Hb S was characterized and electrophoresis came into widespread use as a screening procedure for hemoglobins, the commoner variants were quickly discovered. C, D and E were found not long after S had been characterized. Hb F has long stood for fetal hemoglobin. The letter designation nomenclature was formalized by Chernoff *et al.* (1953). In 1955, Allison suggested that *Hb* be the gene locus name for hemoglobin, but this usage is not followed because of the multiple polypeptide chain structural loci controlling hemoglobin. By the time the letter "Q" was reached, it was apparent that there were not going to be enough letters in the alphabet. Hemoglobins began to be named after places, hospitals or people. Some exotic names were proposed, such as 'Aida', 'Riverdale-Bronx' and 'Abraham Lincoln'. Some of the variants acquired many names, because they were discovered and rediscovered all over the world. It was not until the substitution or chemical alteration was clarified that the identities became apparent. In these cases, the name given by the first observer usually prevails.

A number of hemoglobin variants are distinguishable by electrophoresis in various buffer systems. New variants have sometimes been designated according to their similar electrophoretic mobility, or their similarity in other properties, to a known Hb, followed by the descriptive name. Thus the original Hb D [β121(GH4) Glu→Gln] is called Hb D Los Angeles, D Punjab, D Chicago, D North Carolina, etc. But there are also Hb D Bushman (β16 Gly→Arg), Hb D Iran (β22 Glu→Gln) and Hb D Ibadan (β87 Thr→Lys). Some of the variants have no capital letters in their designations: Hb Sawara (α6 Asp→Ala), Hb Winnipeg (α75 Asp→Thr), Hb Deer Lodge (β2 His→Arg), Hb Alabama (β39 Gln→Lys), etc.

The latest recommendations for standardizing the Hb variant nomenclature (Recommendations, 1979) cover a number of different aspects, some of which have been pointed out in the foregoing sections. For the major normal hemoglobins, Hb A ($\alpha_2\beta_2$) and Hb F ($\alpha_2\gamma_2$) are used. Designations such as A_0, A_{II}, F_0, and F_{II} are discouraged. The embryonic hemoglobins are Gower-I ($\zeta_2\epsilon_2$), Gower-II ($\alpha_2\epsilon_2$) and Portland-I ($\zeta_2\gamma_2$). The only letter designations recommended for the abnormal hemoglobins are Hb C, Hb E, Hb S and Hb H. The remainder should have the descriptive name following the letter, and many variants will have only a descriptive name. Previously, it was the practice to show the alteration as a superscript. Thus, Hb G Georgia was $\alpha_2{}^{95(G2)Pro\rightarrow Leu}\beta_2$, while Hb S was $\alpha_2\beta_2{}^{6(A3)Glu\rightarrow Val}$, and so forth. It is now recommended that only the variant chain, residue number (sequential and helical) and the amino acid substitution be shown, without the use of superscripts. Thus, Hb G Georgia = α95(G2)Pro→Leu and Hb S = β6 (A3)Glu→Val. The designation "Hb M" is retained for abnormal hemoglobins that have an increased tendency to methemoglobin formation, e.g. Hb M Boston = α58(E7) His→Lys. The deletion mutants should signify which residues are "missing", e.g. Hb Freiburg = β23(B5) missing. Fusion hemoglobins are designated so that the segments referring to the types of chains are identified, e.g. Hb Lepore-Boston = δ(1-87)β(115-146). The elongated variants are denoted by specifying the chain, the number of additional residues found at the carboxy-terminus (C), and the residue immediately following the normal C-terminus, (i.e. position 142 in α and 147 in β). Thus Hb Constant Spring = α + 31C (142Gln). Hb H is retained for β_4, and Hb Bart's for γ_4.

38.3 Biochemical Genetics of Hemoglobin

As has been noted, hemoglobin variants have provided a model system for human biochemical genetics. Now that the genetic code is known (Table 1.4), it is possible to speculate intelligently about the kinds of base sequence changes in DNA that could be responsible for the single amino acid substitution variants. Indeed, this kind of analysis can help in the understanding of the evolution of mutations in the human species (see, for example, Beale and Lehmann, 1965; Shaw *et al.*, 1977; and Vogel, 1969).

In addition, studies of the Hb variants have yielded much information about the molecular structure of the polypeptide structural genes themselves. It has been clear for some time that the α and β genes were on different chromosomes (Diesseroth *et al.*, 1976). It is now clear that the α gene is on chromosome 16, while the β and γ gene loci are on chromosome 11 (Diesseroth *et al.*, 1977 and 1978).

There is growing evidence that the α chain locus is duplicated in most people (Nute, 1974; Forget, 1979). It is also quite clear now that there are two γ gene loci per haploid set of chromosomes, one locus coding for the γ136Gly ($^{G}\gamma$) chain, and the other coding for the γ136Ala ($^{A}\gamma$) chain (Schroeder *et al.*, 1972; Schroeder and Huisman, 1974). This point was discussed in section 38.2.2. The chains can be present in different proportions in Hb F, suggesting further complexity at the genetic level (Huisman *et al.*, 1972). The so-called $^{T}\gamma$ chain, reported by Ricco *et al.* (1976) and discussed in section 38.2.2, has γ75Thr instead of γ75Ile. Schroeder *et al.* (1979) regard $^{T}\gamma$ as the product of a modified $^{A}\gamma$ locus and said that the chain should be called $^{TA}\gamma$. Thus, it appears that most individuals have a duplicate set of α genes on chromosome 16, and a series of closely linked γ, δ, and β genes on chromosome 11 (Schroeder and Huisman, 1974; Deisseroth *et al.*, 1977 and 1978). The organization of the chromosome 11 genes is thought to be $^{G}\gamma^{A}\gamma\delta\beta$ (Little *et al.*, 1979). This arrangement helps in understanding how control of the fetal-to-adult "switch" may come about at the level of DNA (Kabat, 1974), although the exact control mechanisms are not yet known (Forget, 1979). In addition, the Lepore type (fusion) hemoglobins and Hb Kenya (see in section 38.2.3.5) can be understood in terms of non-homologous crossovers between $\delta\beta$ regions (Lepore) or between $\gamma\beta$ regions (Kenya) because of misalignment of sister chromatids at meiosis.

Until recently, it was assumed that the information base sequence in DNA was colinear with that of the m-RNA, and that the m-RNA sequence was, in turn, colinear with the amino acid sequence of the protein (section 1.2.2). Recent fine structure studies have revealed the extraordinary fact, however, that the coding sequences of DNA are commonly interrupted by intervening sequences of DNA of variable length (introns) which are not represented in mature m-RNA. The sequences are transcribed initially into precursor m-RNA, and the m-RNA molecules then have these sequences removed enzymatically by a splicing process before the m-RNA is transported to the cytoplasm from the nucleus. Introns have been identified in the non-α chain coding regions of the human genome. The biochemical genetics of hemoglobin synthesis was recently reviewed by Forget (1979) and by Bank *et al.* (1980).

In his remarks opening the major hemoglobin conference at the New York Academy of Sciences in 1974 (Kitchin and Boyer, 1974), Prof. Motulsky summarized the importance of hemoglobin research to modern biochemical genetics (Motulsky, 1974):

> Hemoglobin research plays a role in human biochemical genetics similar to that of drosophila research in formal genetics and analogous to work with microorganisms in microbial genetics. Many fundamental concepts have become clarified by investigations on human hemoglobins.

38.4 Methods of Separating and Characterizing Hemoglobins

The most important method of separating normal and common variant hemoglobins is electrophoresis. Dozens of different electrophoretic procedures have been devised over the years for this purpose. Because some of the variant hemoglobins have clinically significant consequences, there have been a number of mass screening efforts in various populations. Apart from their medical value in identifying people who may require treatment, or who may be advised to seek genetic counseling, many variants have been identified through these studies. As has been noted, these variants have been characterized and are of considerable biochemical genetic significance. Population distributions of the more widely occurring variants are of anthropological and forensic interest as well. Although most hemoglobin variants are rare, it has been estimated that 1 in every 2,000 persons carries a detectable Hb variant (Motulsky, 1974).

The oldest electrophoretic procedures employed paper or starch blocks as support media. Procedures using agarose, CAM, and polyacrylamide gels have since been devised. Some workers still prefer paper electrophoresis (Lehmann and Huntsman, 1974). Most forensic serologists probably utilize cellulose acetate membranes or starch or agarose gels for Hb separation. Isoelectric focusing techniques have also been used to separate Hb variants.

Depending upon which hemoglobins are to be separated, different buffer systems are used. Hemoglobins which coelectrophorese in one system may separate in another. It is sometimes necessary, therefore, to run selected samples in more than one system to distinguish those types that have the same mobility in the first system. Three "basic" buffer systems are used for paper (and most other kinds of) electrophoresis, although dozens of minor variations have been introduced. These are a barbital buffer, pH 8.6–8.9, a Tris-EDTA-borate buffer, pH 8.6–8.9 and a phosphate buffer, pH 6.5. The alkaline buffers give good separations of Hb A, S and C. The Tris system resolves Hb A_2 much better than barbital. Hb A and Hb F are not well resolved in these buffers; neither are C and E, nor S and D. At pH 6.5,

all the hemoglobins migrate cathodically except Hb H. Paper electrophoresis is discussed in detail by Weiss (1968) and by Lehmann and Huntsman (1974).

Starch gel electrophoresis is somewhat more cumbersome and time-consuming than CAM electrophoresis. For many applications, barbital or Tris-EDTA-borate buffers in the pH 8.6–8.9 range are used. The relative electrophoretic mobilities of a number of hemoglobins in pH 8.6 buffers are indicated in Figure 38.4. Starch gel electrophoresis of hemoglobins has been reviewed by Huehns (1968), and cellulose acetate membrane techniques are given by Chin (1970). In 1957, Robinson *et al.* proposed using agarose gel electrophoresis in acidic (sodium citrate, pH 6) buffer for Hb separation. The system gave a good resolution of Hb A and Hb F, and additionally differentiates Hb S from D and Hb C from E. Relative electrophoretic mobilities of some hemoglobins in acid citrate buffered agarose are shown in Figure 38.5. Agarose gels have been used at alkaline pH as well (Lepp and Bluestein, 1978). Breen *et al.* (1968) reported improved resolution of hemoglobins on cellulose acetate with the Beckman Microzone system using Tris-glycine, pH 9.3, buffers. Jacobson and Vaughan (1977) reported a rapid starch gel procedure for mouse hemoglobins. Schneider and Hightower (1977) have studied the behavior of dozens of different hemoglobins on agarose gels using citric acid buffers at acid pH.

In 1977, Burdett and Whitehead used polyacrylamide gel isoelectrofocusing to separate hemoglobins in the pH 5–8.5 range. Hb A, A_2, S, F, D, C and E could be resolved in this system. In 1978, Bassett *et al.* applied PAGIF in the pH range 6 to 9 to the study of about 70 different Hb variants. They obtained good resolution of many of them, and said that the technique provided a good screening procedure if very thin gels were used in order to conserve the costly ampholines.

Electrophoresis is the method of choice for Hb screening in most laboratories. CAM methods are popular because they are so fast and require very small amounts of material if microtechniques are used. Identification of Hb A, A_2, S, F, D and E can be accomplished fairly readily by electrophoresis. It is necessary to use different electrophoretic techniques for certain of these. Samples running like Hb S at pH 8.6, for example, can be run in citrated agarose to see whether Hb D is present. Identification and characterization of other hemoglobins normally requires more sophisticated techniques. PAGIF is a good approach to Hb separation in laboratories equipped for it. If it is necessary to diagnose the amino acid replacement in a variant Hb, peptide "fingerprinting" and sequencing techniques must be used. Peptide fingerprinting is discussed very clearly by Lehmann and Huntsman (1974), as is the use of amino acid analyzers. Cohen-Solal *et al.* (1974) discussed a specialized sequencing procedure. Recently, Garver *et al.* (1977) have employed RIA techniques to the identification and quantitation of several variant hemoglobins. It is clearly necessary to have the monospecific antiserum in order to use this procedure, so it is useful for variants which have already been characterized. The antisera, however, are specific for the amino acid substitution in the chain, and are, therefore, extremely specific if a mixture of hemoglobins is being investigated.

38.5 Medicolegal Applications

38.5.1 Disputed parentage

No references to the application of Hb variants as such to parentage problems were found, and it does not appear that hemoglobin is widely used for this purpose. According to a recent survey of laboratories in this country (Polesky and Krause, 1977), about 25% of the 30 AABB Reference Laboratories could do Hb typing in the cases, and somewhat fewer did it routinely. About 17% of the other laboratories said that Hb typing was available, but less than 2% used it routinely. The main reason for using Hb typing would be detection of Hb S in cases involving Black subjects. The probability of excluding a falsely accused Black father on the basis of Hb S typing is about 4.5% (Chakraborty *et al.*, 1974). The value would be less for Hb C and Hb D, since they are less common. Hb E would be a useful parentage marker in certain Asian populations.

38.5.2 Hb Typing in bloodstains

For practical purposes, the hemoglobins that might be diagnosed in forensic cases are A, F, S and C. Hb D and E can be separated from Hb S and C under certain electrophoretic conditions. Investigations involving young children may call for Hb F differentiation. This may be achieved in a number of ways, including immunological, electrophoretic, and by alkali denaturation characteristics. This subject was discussed in section 8.3.1. It would, of course, be informative to find significant amounts of Hb F in an adult's blood, since this condition is comparatively rare. Hb F can be distinguished from Hb A in three ways: (1) differential alkali denaturation behavior; (2) immunologically; and (3) electrophoretically. The fact that Hb A is considerably more alkali-labile than Hb F was noted by Körber (1867), and studied in detail by Singer *et al.* (1951). This method of differentiation was applied to bloodstains by Culliford (1964). He followed the denaturation in the visible region. Watanabe (1969) said that the reaction could be followed in the UV. A description of the procedure is given in Culliford (1971), where he says that caution should be exercised in trying to differentiate Hb F by this method in bloodstains more than about a week old. The technique is also described in the most recent methods list from the London laboratory (MPFSL, 1978). Apparently, it is primarily a back-up for electrophoretic or immunological techniques.

Immunological procedures for the differentiation of Hb A and Hb F depend upon having comparatively specific anti-human Hb for the purpose. Efforts to prepare anti-Hb F have been going on for many years (Darrow *et al.*, 1940; Ikin *et al.*, 1953), and antisera to human Hb was first prepared by Klein (1904, 1905a and 1905b), following the work of Leblanc (1901) and Ide (1902) on animal hemoglobins. This subject was discussed in sections 7.1 and 8.3.1. Anti-Hb F procedures have been developed for bloodstains

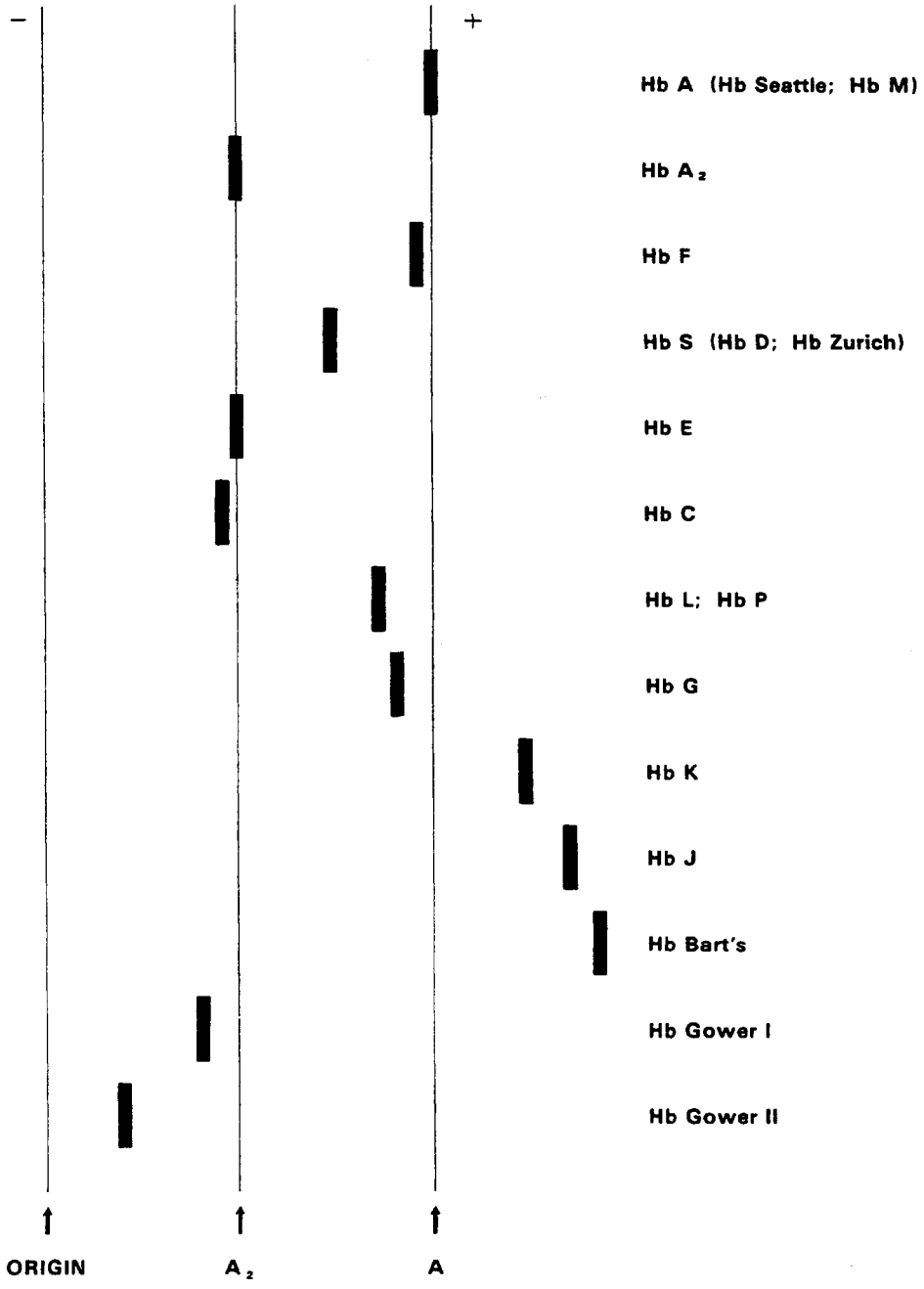


Figure 38.4 Relative Electrophoretic Mobilities of Some Hemoglobins at pH 8.6

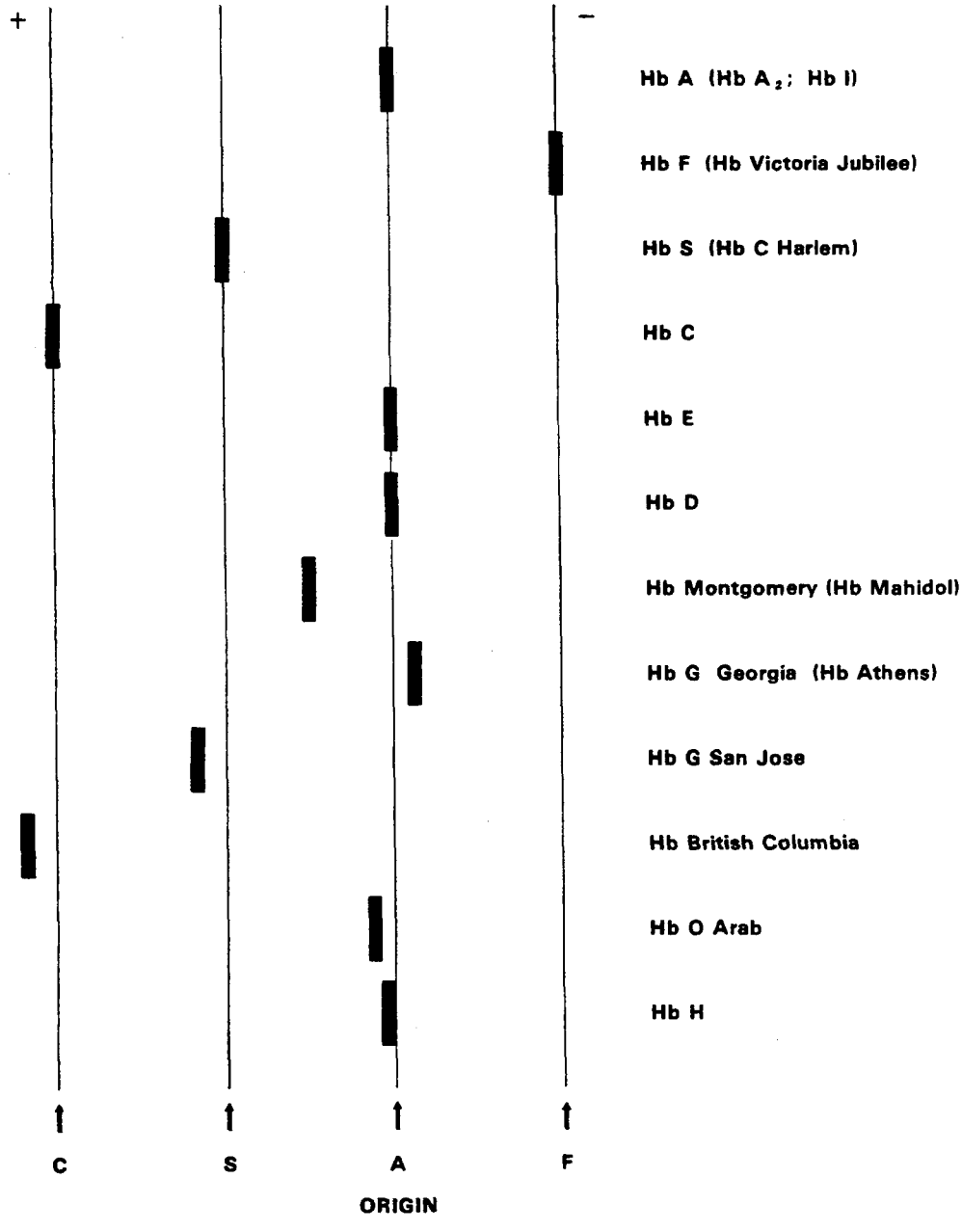


Figure 38.5 Relative Electrophoretic Mobilities of Some Hemoglobins of pH 6 in Agarose

using immunoelectrophoresis (Depieds *et al.*, 1960), Ouchterlony double diffusion, and crossed over electrophoresis (Baxter and Rees, 1974a; MPFSL, 1978; Wiggins and Wraxall, 1979).

Electrophoresis is the favored procedure for separating, and thereby identifying, not only Hb A and Hb F, but also C, S, D and E. Pollack *et al.* (1958) first suggested the application of Hb typing to bloodstain cases. They used paper electrophoresis, and could differentiate Hb A and Hb S in bloodstains on a robbery victim's clothing in a case in which the suspected assailant was Hb AS. They suggested that A and F might be separable as well. Hb A and F do not separate very well in the classical alkaline (pH 8.6–8.9) buffer systems. In 1972, Wraxall reported that good A and F separation could be obtained using barbital pH 8.6 cathodic tank and Tris-EDTA-borate pH 9.1 anodic tank buffers. This system was also applicable to A-S and A-C separations and is described in Culliford (1971). Wilkins and Oepen (1977) confirmed Wraxall's (1972) findings. In 1962, Huntsman and Lehmann applied paper electrophoresis to the separation of Hb A, S, C, D and E in bloodstains a few days old. Hb D and F could be differentiated by alkali denaturation, they said. Culliford (1964) used the discontinuous buffer system of Poulik (1957) to separate Hb A from S and C on Oxoid CAM. The procedure devised by Wraxall (1972) was adopted later (Culliford, 1971), and Sartorius CAM's were preferred. Recently, Wiggins (1978) applied the acid citrate agarose electrophoretic method to the differentiation of Hb S from D and Hb C from E in dried blood. Using this procedure, Hb can be run in the alkaline system (Wraxall, 1972) to distinguish A, F, S and C, and a series of rare variants (MPFSL, 1978). The Hb AS or S, and AC or C samples are then checked in the citric acid agarose system to distinguish S from D and C from E, and thus giving far better population discrimination when the rarer types are encountered. The way this technique works may be better appreciated in a general way by comparing Figures 38.4 and 38.5. Baxter and Rees (1974b) described Hb typing in conjunction with their routine Hp typing procedure (section 39), and said that A, S, C and D could be distinguished. Hb F could be presumptively identified as well.

Hb typing by electrophoresis is usually quite straightforward, provided the limitations of the techniques are recognized and suitable Hb standards are available. In 1979, Barnard and Grunbaum looked at a series of Hb A, AS and AC stains up to 28 days old, however, and noted that aqueous extracts of the bloodstains could exhibit altered electrophoretic mobility relative to the standard hemolysates. The effect was reversible by extracting the bloodstain with Cleland's reagent (DTT). Reversal of the observed effects by DTT strongly suggests that these alterations were based on sulfhydryl effects of some kind. Some years ago, Huisman *et al.* (1966) noted that Hb A, S and F showed similar effects upon 4° storage of hemolysates. Dialysis tended to prevent the effects, while reduced glutathione tended to enhance them. These workers thought that the storage effects could be understood in terms of mixed disulfide formation between glutathione and globin chains. These observations may be related to those of Barnard and Grunbaum (1979).

Most forensic science laboratories are not speciality hemoglobin laboratories. Differentiation of Hb A, F, S, C, D and E can be accomplished fairly readily. Beyond that, though, known standards are unlikely to be available. There are now hundreds of variants of Hb, and most of them are not readily distinguishable by electrophoresis. More complicated characterization techniques, such as peptide fingerprinting and sequence analysis, are beyond the scope of most forensic laboratories. In addition, fairly large samples are required which may not be available, and it is not completely clear that Hb isolated from dried blood is amenable to such analysis. Thus, while one might diagnose a sample as AC or AS or AD according to its electrophoretic mobility, there is a possibility that the sample is really a rare variant with a comparable mobility. While the majority of variants are indeed rare, it would not be surprising for a fairly busy casework laboratory that types Hb routinely to encounter one of them on occasion. According to Motulsky (1974) about 1 in 2,000 people has a detectable variant hemoglobin. If a rare variant were encountered, and could be identified (perhaps with the help of specialized Hb laboratory), it would be highly individualizing and informative.

38.6 Distribution of Common Hemoglobin Variants in U.S. Populations

The data are shown in Table 38.2. The most common variant hemoglobin is probably Hb S. The population genetics of Hb S, and by analogy to what is known of other Hb variants, suggest that the mutation causing S has occurred more than once. There is not much doubt that West Africa represents at least one point of origin. Hb S has apparently reached polymorphic proportions in areas of the world in which malaria was (or is) endemic. Heterozygotes (Hb AS) have an advantage against malaria, and this situation has been called "balanced polymorphism". The gene exists in other parts of Africa, in India and other parts of Asia, and in parts of Europe. The gene may now be seen in any population into which there have been any significant migrations from high S regions. In this country, Hb S is largely, but not exclusively, restricted to Blacks of African origin. Hb S may be seen in Asians, and it is occasionally seen in Whites (e.g. Crane *et al.*, 1977). It is not clear from most of the reports of Hb S in Whites whether the hemoglobin has been rigorously identified as the β6Glu→Val molecule, or whether a different variant which looks like S electrophoretically could be present. It is, of course, possible that Hb S that is β6Glu→Val arises periodically in a family by spontaneous mutation. Hb D was first described in Los Angeles, but occurs in significant frequencies in certain Asian Indians. It is also seen in Pakistan, Iran and Afghanistan. It is seen in Europeans, particularly those from countries which had close ties with India, and it has been noted that Hb D was most common in this country in people who were partly of English origin. Hb C was

detected in American Blacks. It is present in West Africa, but almost absent in East Africa. It is also seen in Europeans upon occasion. There is recent evidence (Kan and Dozy, 1980) that the S and C mutations arose independently, and that Hb AS arose by several independent mutational events in different places. This conclusion has been reached by studying the relationship of Hb S and C to a polymorphism for a restriction endonuclease recognition site on DNA, adjacent to the β chain structural gene. Hb E is principally an Asian type, being found at appreciable frequencies in Burma and Indochina. It is not common in India nor in modern Chinese. Lehmann and Huntsman (1974) provide a thorough discussion of the world population distributions of various hemoglobins. The best and almost only comprehensive reference source to population frequencies is Livingstone (1967).

Table 38.2 Distribution of Common Hb Phenotypes in U.S. Populations

Population	Number	Phenotypes — Number (Percent) A	AS	S	AD	D	AC	C	Reference
CAUCASIAN									
Baltimore, MD	500	500 (100)							Smith and Conley, 1953
Ann Arbor, MI	72						(0)		Neel, 1954
Houston, TX	350	350 (100)							Haynie et al., 1957
Durham, NC ("non-Black")	734	732 (99.7)	1 (0.14)		1 (0.14)				Chernoff and Welchselbaum, 1958
Southern Louisiana (children)	140	139 (99.3)	1 (0.7)						Moffitt and McDowell, 1959
Memphis, TN area (autopsy)	1,250	1,250 (100)							McCormick, 1960
St. Louis, MO (infants)	90	90 (100)							Minnich et al., 1962
Baltimore, MD (infants)	180	180 (100)							Weatherall, 1963
Mississippi	1,045	1,044 (99.9)		1 (0.1)					Thompson et al., 1964
California	6,004	(99.8)	(0.2)				(0)		Grunbaum et al., 1978
Detroit, MI	503	503 (100)							Stolorow et al., 1979
California	1,040	1,040 (100)							Grunbaum et al., 1980
NEGRO									
Baltimore, MD	500 ★	449 (89.8)	36 (7.2)	5 (1)			9 (1.8)		Smith and Conley, 1953
Ann Arbor, MI	209	(98.6)					(1.4)		Neel, 1954
St. Louis, MO	1,020	896 (87.8)	94 (6.3)		4 (0.4)		26 (2.5)		Chernoff, 1956
Galveston, TX (patients)	1,550	1,369 (88.3)	141 (9.1)	4 (0.3)			35 (2.3)	1 (<0.1)	Schneider, 1956
Houston, TX	400 ★	351 (87.8)	36 (9)	5 (1.3)			6 (1.5)	1 (0.25)	Haynie et al., 1957
Durham, NC	390	338 (86.7)	33 (8.5)		1 (0.25)		13 (3.3)		Chernoff and Welchselbaum, 1958
Southern Louisiana (children)	564 ☆	479 (84.9)	47 (8.3)	18 (3.2)			10 (1.8)		Moffitt and McDowell, 1959
Puerto Rico	602 ○	561 (93.2)	29 (4.8)	2 (0.3)			7 (1.2)	1 (0.2)	Suarez et al., 1959
Philadelphia, PA (patients)	1,000 ○	895 (89.5)	74 (7.4)	3 (0.3)	1 (0.1)		23 (2.3)		Myerson et al., 1959
Baltimore, MD	400 □				4 (0.4)				Marder and Conley 1959
Memphis, TN area (autopsy)	2,800 ■	2,459 (87.8)	254 (9.1)	19 (0.7)	1 (<0.1)		60 (2.1)		McCormick, 1960

Table 38.2 (Cont'd.)

Population	Number	Phenotypes — Number (Percent) A	AS	S	AD	D	AC	C	Reference
Washington, D.C. (tuberculous)	310	282 (91)	28 (9)						Ryan et al., 1960
St. Louis, MO (infants)	449◇	359 (79.9)	47 (10.5)		2 (0.4)		9 (2)		Minnich et al., 1962
Wash. D.C. (pregnant women)									
Study group	524	490 (93.5)	25 (4.8)	1 (0.2)			8 (1.5)		Jenkins and Clark, 1962
Control group	304	283 (93.1)	11 (3.6)				10 (3.3)		
Baltimore, MD (infants)	900◆	784 (87.1)	67 (7.4)				26 (2.9)		Weatherall, 1963
Maryland	681	625 (91.8)	44 (6.5)				12 (1.8)		Boyer et al., 1963
Southeast Georgia	237	214 (90.3)	19 (8)				4 (1.7)		Cooper et al., 1963
Gainesville, FL (pregnant women)	944	869 (92.1)	65 (6.9)	1 (0.1)			9 (0.95)		Cotter and Prystowsky, 1963
Mississippi	1,310	1,100 (84)	114 (8.7)	37 (2.8)	14 (1)		38 (2.9)	7 (0.5)	Thompson et al., 1964
Southern Louisiana (tuberculous)	220	211 (95.9)					9 (4.1)		Coulter, 1965
Alabama	249,089●	220,405 (88.5)	21,423 (8.6)	574 (0.2)			6,074 (2.4)	102 (0.04)	Schneider et al., 1976
California	1,025○	(89.3)	(8.6)				(1.8)		Grunbaum et al., 1978
Detroit, MI	504▲	452 (89.7)	37 (7.3)				14 (2.8)		Stolorow et al., 1979
California	792△	716 (90.4)	54 (6.8)				16 (2.3)		Grunbaum et al., 1980
HISPANIC									
Puerto Rico ("White")	1,487	1,486 (99.9)	1 (0.06)						Suarez et al., 1959
California, (Chicano/Amerindian)	1,596●	(99.6)	(0.1)				(0.2)		Grunbaum et al., 1978
California (Mexican)	1,569○	1,561 (99.5)	3 (0.2)				3 (0.2)		Grunbaum et al., 1980
ASIAN									
California/Hawaii	3,053●	(99.9)							Grunbaum et al., 1978
California/Hawaii	1,451○	1,448 (99.8)	1 (0.06)						Grunbaum et al., 1980

★ one SC
☆ ten SC
✪ two SC

□ those classified as AS by electrophoretic screening were tested further

■ four SC and three other
◇ 32 other
● 23 other

● 149 of the "S" were S/β+ thal, 73 were S/high F; 329 were S/C. 11 were S/other and 7 were C/other; 164 were rare variants

○ 0.4% were rare
▲ one rare
△ four rare

● 0.1% rare
○ two rare

SECTION 39. INTRODUCTION TO SERUM (PLASMA) PROTEINS

Various polymorphic serum protein (serum group) systems will be considered in subsequent sections (40 through 45) of this unit, and a brief introduction to serum proteins is appropriately included here. Some of the enzymatic activities considered in Unit VI occur in serum, and they can be considered "serum proteins" as well. Any polymorphic system which exhibits enzymatic activity has been included in the previous unit of this book, however. The serum proteins and serum group systems included in Unit VII do not possess known *in vivo* enzymatic activities, but are defined by a number of other properties (often immunological, serological or electrophoretic). Some of these proteins are not yet understood in terms of physiological function. The terms "serum protein" and "plasma protein" are used interchangeably in what follows.

The development of various, reproducible protein separation techniques which complement one another in terms of resolution and specificity, has enabled investigators to separate, classify and characterize many of the proteins of human plasma (as well as thousands of other proteins). Human plasma possesses a large number and variety of proteins, some of which are known to be under the control of polymorphic genetic loci. A number of the polymorphic serum protein systems may be utilized in forensic and medicolegal investigations.

The nomenclature of plasma proteins is rather complex and cumbersome, because it incorporates a number of historical classification designations along with the refinements that have been introduced as separation techniques improved and the proteins have been better characterized. There has never been a coordinated international effort to arrive at a truly systematic nomenclature, such as was done for the enzymes by the Enzyme Commission (see section 1.1.3.2). Plasma proteins were first classified according to their solubility in water and salt solutions, then grouped into classes according to electrophoretic mobility at a restricted pH, defined in terms of cold ethanol solubility, and eventually resolved by various kinds of electrophoresis, immunoelectrophoresis and electro-focusing techniques into various components, not all of which have yet been identified with a particular function. An older review of serum proteins, primarily of historical interest, may be found in Howe (1925). As happens repeatedly, some of the proteins have been identified in a number of different laboratories, and have been given a variety of names. In most cases, one of the names gains widespread acceptance. The nomenclature of certain classes of plasma proteins (such as the immunoglobulins) has been standardized by international agreement. Some effort has been made to arrive at a uniform nomenclature for the genetic variants of serum proteins, and this has been partially successful. This matter is considered in subsequent sections in connection with particular systems.

The question of what actually constitutes a serum (plasma) protein is not that easy to answer. Some criteria can be established, but there are always proteins that are difficult to classify, in part because they are poorly characterized and/or have no known function. Putnam (1975a) distinguished between the "true" serum proteins, and what were called "passenger proteins". The latter may be found in the serum at certain times, or even all the time, but they traverse the serum in transit from one site to another as the result of a particular physiological condition (e.g. pregnancy), as the result of disease, or because they are directly involved in transport processes. No classification system can easily accommodate the number and variety of plasma proteins now known. There are some protein systems in plasma whose members act together in a concerted manner, such as the complement system, the lipoproteins, the coagulation proteins, the protease inhibitors and the immunoglobulins. There are about 35 to 50 proteins generally recognized as true plasma proteins; but there are probably 100 or more other proteins which can be found in plasma, but have not been well characterized.

Putnam (1975a) presented several criteria for classifying a protein as a "plasma protein": (1) The protein must be present in plasma after the neonatal period; (2) Synthesis must take place in the liver or the reticuloendothelial system; (3) The primary function (if known) must be mediated in the vascular system, rather than in the target system; (4) The protein should be actively secreted into the bloodstream, and not be there as a result of tissue damage or capillary permeability; (5) The concentration should be higher in blood than in other fluids, except in the specialized cells which synthesize the protein; (6) It should have an appreciable half-life in plasma, and not be transitory; (7) Genetic polymorphism (if exhibited) should not be traceable to tissues of origin, as is the case with a number of the enzyme systems (see in Unit VI); and (8) A true plasma protein is not derived by proteolytic cleavage or catabolism of other plasma proteins, as for example the Fab and Fc fragments of immunoglobulins; however, true precursors, and their active forms, e.g. plasminogen and plasmin, should both be considered plasma proteins.

It is difficult to arrive at a criterion based upon minimal concentration in plasma, because the plasma proteins exhibit a very wide range of concentrations. Albumin is present in concentrations of about 5 g/100 mℓ, while the immunoglobulin IgE occurs to the extent of about 5×10^{-7} g/100mℓ. On the basis of concentration in normal

plasma, one can arbitrarily establish four classes of serum proteins (Putnam, 1975a): Predominant proteins, such as albumin and IgG (1–5 g/100 mℓ); Other major proteins (100–1000 mg/100 mℓ); Minor proteins (10–100 mg/100 mℓ); and Trace proteins (< 10 mg/100 mℓ). The lower the concentration of a protein, the more sensitive must be a technique used to measure its presence, and the less likely that normal concentration ranges have been established. Some of the proteins occurring at very low normal concentrations have been studied because their levels are greatly elevated for various reasons in certain individuals. A list of some of the better characterized serum proteins, and some of their properties, is given in Table 39.1. The properties of many of these proteins have been reviewed by Putnam (1975b) and by Cooper (1978).

The serum proteins which exhibit genetic variation at polymorphic levels constitute an important class of genetic markers. They are discussed in more detail in the sections which follow. A review of the properties of a number of polymorphic serum proteins was given by Gitlin and Gitlin (1975). Genetic variation in a protein may be reflected in the protein's structure itself, or in the amount of the protein that is present. Both structural and quantitative variation may have a number of different genetic bases. The principles of biochemical genetics that were outlined in section 1.2.2, that were seen to operate in the case of isoenzyme variation (Unit VI), and about which a great deal has been learned from the study of Hb variants (section 38), are fully applicable to plasma proteins. Alterations, additions or deletions of bases in the coding sequence for a protein may lead to an altered structure for that protein which is detectable and recognizable. The alteration can be a single amino acid substitution, or a much more drastic one. Deletions or additions of bases in the coding sequence can cause a shift in the "reading frame", and alter the structure of the protein beyond the point of occurrence. The amino acid sequence may be different, or the polypeptide chain can be shortened if a nonsense or termination codon was introduced. Unequal crossovers between genes can result in polypeptide chains much shorter or longer than is usual. Quantitative changes are often genetically controlled as well, although the mechanisms may be indirect. Alterations in genes controlling products responsible for conversion of one protein to another, alterations in operator or regulator genes which control structural genes, or alterations in the production of m-RNA can all give rise to various quantitative variations. The number and variety of genetic variants known for a particular system are dependent upon the likelihood with which they will come to someone's attention, and upon the ability of the various techniques to discriminate them. Subtle, or even not so subtle, variations which have no clinical manifestations or implications are less likely to be noticed than those which do have. Similarly, even in cases where various populations are screened for variants or abnormalities of a certain protein, the technique being used is unlikely to be able to detect every kind of variant. Electrophoresis, for example, readily detects variants in which the net charge of the protein is altered. But the substitution of one uncharged, straight chain amino acid for another, for example, could easily escape electrophoretic detection. A one base change in a gene leading to a GUU codon instead of a GCU codon would lead to a Ala→Val substitution in the polypeptide chain for example.

The development of many of the methods used for the study of serum proteins was discussed in section 2. In many ways, the nomenclature of the serum proteins has grown up and developed along with the methods used to study them. In the last century, the terms "albumin" and "globulin" were coined to represent serum protein fractions that were soluble and insoluble, respectively, in water. With the development of salt fractionation techniques, different "globulins" were distinguished (euglobulins; pseudoglobulins). An account of the many studies conducted, and the terms that were introduced, may be read in Pedersen (1945). Development of moving boundary electrophoresis (Tiselius, 1930) by Tiselius and his collaborators was an important step. Using the technique, four distinct fractions could be distinguished in horse serum (Tiselius, 1937), and these were named α, β and γ globulin components. The fourth fraction was albumin. Development of analytical ultracentrifugation, and its application to the study of serum proteins (see Pedersen, 1945), was also an important step in the effort to characterize the various components more completely. Subsequent electrohoretic investigations led to subdivision of the original serum protein fractions: α_1, α_2, β_1, β_2, γ_1, γ_2. The subscript notation is traditional, but it is equally acceptable today to write α1, α2, β1, etc., or α-1, α-2, β-1, etc. (Putnam, 1975a). These electrophoretic characterizations of serum proteins are still used to place many of them into generic classes, even though it is recognized that any number of structurally and functionally diverse proteins may be under a single electrophoretic peak. Development of zone electrophoretic, immunoelectrophoretic and isoelectrofocusing procedures, as well as immunofixation detection techniques and their variations, have accelerated the flow of new information about various serum proteins. Zone electrophoresis was first performed on paper as a supporting medium (Cremer and Tiselius, 1950; Kunkel and Tiselius, 1951), and later on starch gels (Kunkel and Slater, 1952). Starch gel electrophoresis was significantly refined by the investigations of Smithies and collaborators (Smithies, 1955a, 1955b, 1959a, 1959b; Poulik and Smithies, 1958), with the characterization of several serum proteins occurring along the way (see in subsequent sections). Resolution could be enhanced by carrying out electrophoresis in two dimensions (Smithies and Poulik, 1956). Electrophoresis may be carried out on cellulose acetate foils (Kohn, 1957 and 1958), agarose gels (Gordon *et al.*, 1949), and polyacrylamide gels (Raymond and Weintraub, 1959) as well. The sensitive and high-resolution polyacylamide disc gel electrophoresis technique was introduced by Ornstein (1964) and Davis (1964). Immunoelectrophoresis is likewise an important and sensitive technique for the separation and characterization of serum proteins (see in Grabar and

Table 39.1 Properties Of Some Serum (Plasma) Proteins

Name of Protein	Synonyms	Symbol	Molecular Weight	Electrophoretic Mobility pH 8.6 Barbital (μ = 0.1)
Prealbumin	ρ_1-protein Prealbumin I Thyroxine-binding prealbumin (TBPA)	PA	55,000	7.6
Albumin	Serum albumin	Alb	68,300	5.9
α_1-acid glycoprotein	orosomucoid α_1-seromucoid α_1-globulin	α_1-S	44,000	5.2
α_1T-glycoprotein	Tryptophan-poor α_1-glycoprotein	α_1T	≅60,000	
Transcortin		TC	55,700	
α_1-antitrypsin	α_1-3.5S glycoprotein α_1-glycoprotein α_1-seromucoide de Schultze α_{1A}-globulin α_{1B}-globulin	α_1-AT (PI)	54,000	5.4
α_1-antichymotrypsin	α_1X-glycoprotein	α_1X	68,000	
α_1B-glycoprotein	easily precipitable α_1-glycoprotein	α_1B	50,000	
Zn-α_2-glycoprotein		Znα_2	41,000	4.2
Thyroxine-binding globulin		TBG	58,000	
Antithrombin III	α_2-antithrombin	ATIII	≅65,000	
Gc-globulin	Group specific component Gc-factor postalbumins 2 + 3	Gc	50,800	
C1s component	C'1 esterase	C1s	88,000	
Inter-α-trypsin inhibitor		IαI	≅160,000	
Retinol binding protein		RBP	21,000	
α_2HS-glycoprotein	Ba-α_2-glycoprotein α_2Z-globulin α_2HS-mucoid postalbumin 3	α_2-HS	49,000	4.2
C1 inactivator	α_2-neuraminoglycoprotein C1 esterase inhibitor	C1-ina	104,000	
3.8S histidine rich α_2-glycoprotein		HRG	58,500	
C9 component		C9	79,000	
Haptoglobin	seromucoide α_2	Hp	100,000 (1-1)	4.6
Ceruloplasmin	α_2-IV Metallosromucoide α	Cp	151,000	4.6
Serum cholinesterase	Pseudocholinesterase	PCE ChE E_1	348,000	3.1
α_2-macroglobulin	Sa$_2$-globulin *seromucoide α_2 de Schultze*	α_2M	725,000	4.2
Plasminogen	Profibrinolysin	Pmg (Pg:PLG)	81,000	3.7
Hemopexin	Heme-binding β-globulin Seromucoide β_{1A} Seromucoid β_{1B} β_{1B}-globulin β_1-haptoglobin (β_{1H})	Hpx	57,000	3.1
Transferrin	Siderophilin β_1-metal combining globulin β_{1S}-globulin	Tf	76,500	3.1
C2 component		C2	206,000	
C3 proactivator	glycine-rich β-globulin	C3PA	≅60,000	
Clr component		Clr	160,000	
C5 component	β_1F-globulin	C5	180,000	
C3 component		C3	185,000	
C4 component	β_1E-globulin	C4	206,000	
β_2-glycoprotein-I	β_{2X}-globulin β_2-mucoid	β_2 I	40,000	1.6
C6 component		C6	95,000	
C7 component		C7	100,000	
IgA	γ A immunoglobulin γ_{1A}-globulin γ_{2A}-globulin	IgA	180,000	2.1
C8 component		C8	153,000	
Fibrin stabilizing factor	Coagulation factor XIII	FXIII	340,000	
IgM	γM immunoglobulin γ_1M-globulin γ_1-macroglobulin 19S γ-globulin β_{2M}-globulin	IgM	1,000,000	2.1
IgG	γG immunoglobulin 7S γ-globulin γ-globulin γ_{SS}-globulin γ_2-globulin	IgG	160,000	1.2
C reactive protein		CRP	135,000	
Porperdin			220,000	
C1q component	11S component	C1q	400,000	
Lysozyme	muramidase		≅15,000	
α_1-lipoprotein	high density lipoprotein			
β-lipoprotein	*low-density lipoprotein*			
Fibrinogen	coagulation factor I		341,000	2.1

Burtin, 1964). The quantitative versions of immunoelectrophoresis (such as rocket electrophoresis and crossed immunoelectrophoresis) yield additional information about a mixture of serum proteins (see Axelsen *et al.*, 1973). One of the problems with many serum proteins is specific detection. Methods making use of specific antibodies are among the most useful for this purpose, and electrophoretic separation may be combined with an overlay detection system that incorporates a specific antibody. The technique is usually called immunofixation electrophoresis (Alper and Johnson, 1969); it was originally devised by Wilson (1964), who called it "direct electrophoresis". Immunofixation electrophoresis is used for the electrophoretic typing of several polymorphic serum protein systems of interest in forensic science. The relationship between various serum proteins as they appear after separation by various forms of electrophoresis and immunoelectrophoresis is shown in Figure 39.1.

Individual polymorphic serum protein systems are discussed in subsequent sections of Unit VII. Attempts to diagnose individual differences in "serum protein profiles" are discussed in Unit VIII.

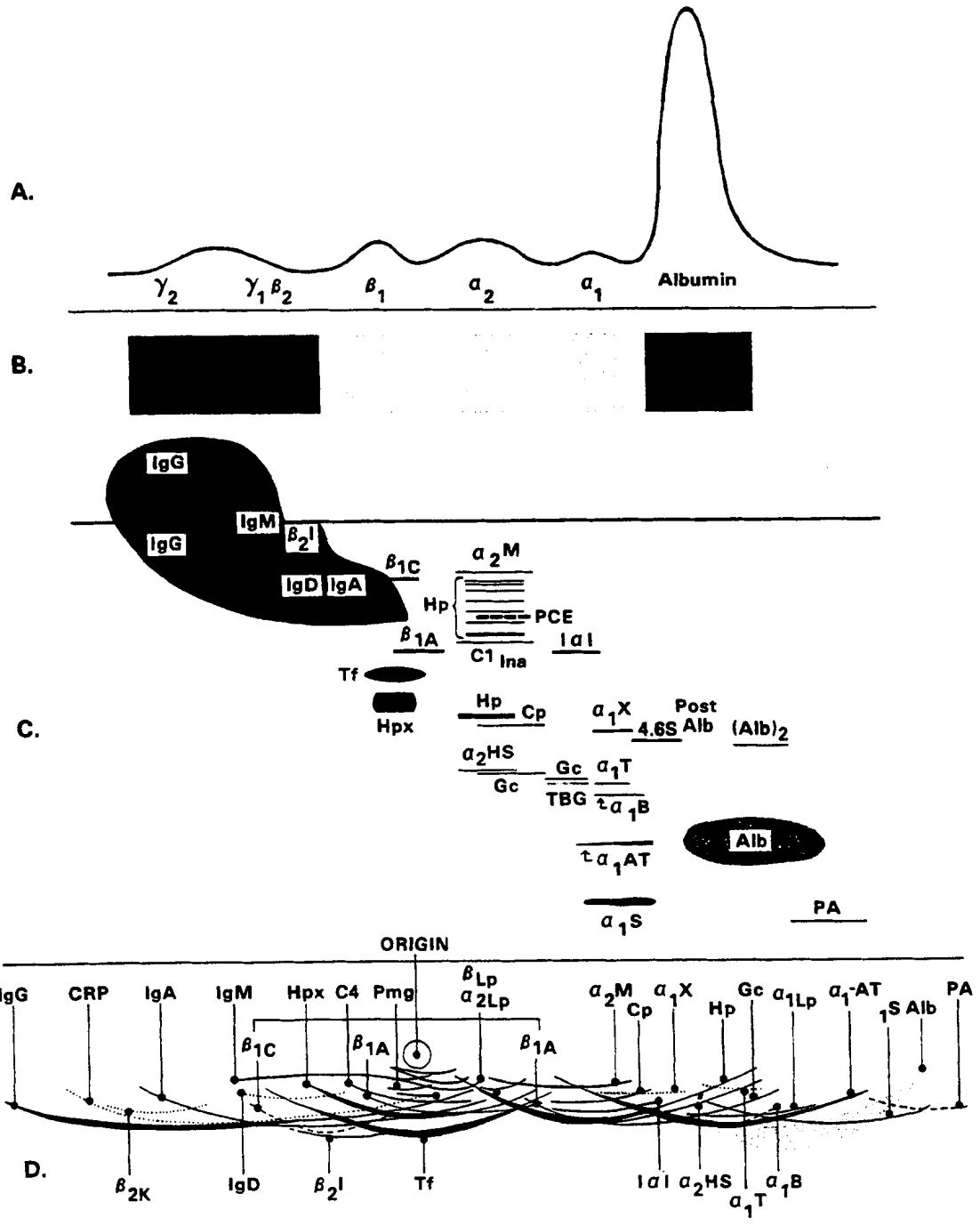


Figure 39.1 Plasma (Serum) Protein Profiles by Various Techniques

A. Moving Boundary Electrophoresis (pH 8.6)
B. Paper Electrophoresis
C. Two-dimensional Starch Gel Electrophoresis
D. Immunoelectrophoresis

SECTION 40. HAPTOGLOBIN

40.1 Recognition of Haptoglobin (Hp)

In 1938, Polonovski and Jayle found a protein in serum which bound hemoglobin. They had noticed that the addition of serum enhanced the peroxidase activity of Hb (section 6 introduction). The Hb binding protein was further characterized, found to be an α_2 glycoprotein, and given the name "haptoglobin", from a Greek root meaning "to bind" (Polonovski and Jayle, 1940). They had considered calling the protein "prosaptoglobin", but had settled on the now familiar name. "Haptoglobin" is not a single molecule, but a group of closely related ones, and it is proper to speak of "the haptoglobins". The multiplicity of molecular forms is now known to be based on the control of Hp synthesis by a polymorphic genetic locus, as a result of which one may refer to the "haptoglobin system". Determination of serum haptoglobin levels was found to be a clinically useful and significant measurement, and standardized procedures based on Hb binding and peroxidase activity (Jayle, 1951; Connell and Smithies, 1959) and using quantitative immunochemical techniques (Kluthe *et al.*, 1965) were developed. It is now clear that the quantitative immunochemical methods are not as straightforward as was first thought (Valette *et al.*, 1979), in that the results obtained are dependent on the Hp phenotype. This behavior can probably be explained by immunodeterminant differences in the Hp subunits (see below). The extensive studies of the Paris group on haptoglobins were well summarized by Jayle and Moretti (1962).

40.2 Haptoglobin Physiology

Many studies have been conducted on haptoglobin physiology and function (Jayle, 1956; Kirk, 1968a; Giblett, 1968 and 1969; Javid, 1967b; Pintera, 1971), and not every recorded observation has been satisfactorily accounted for as yet. The liver is probably the site of haptoglobin synthesis, and the amount of Hp produced is subject to many influences. It can vary considerably in different physiological and pathological states. There is some variation in the normal level of Hp, values being of the order of 40 to 180 mg/100 mℓ, expressed in terms of Hb binding capacity. The quantity of Hb bound by Hp is related to the Hp type (see below). Haptoglobin may be catabolized as free Hp, or in complex with Hb. More is known about the latter route. Free hemoglobin released into circulation, is immediately complexed with Hp, and the complex is removed by the reticuloendothelial system. The half-life of the complex in circulation is a function of its concentration (Noyes and Garby, 1967), the clearance rate tending to be more exponential at lower concentrations of complex, and more linear at the higher ones.

Free hemoglobin in plasma can pass across the renal glomerulus, whereas Hb complexed with Hp cannot (Allison and ap Rees, 1957). As a result, hemoglobin is cleared from plasma (at least in rabbits) significantly faster than is Hb–Hp complex (Murray *et al.*, 1961). However, Hb is found to be cleared more quickly even in nephrectomized animals. In 1958, Allison suggested that a principal function of Hp was to prevent the loss of Hb (and, hence, the loss of iron) across the renal glomerulus. Both free Hb and Hb–Hp complex are catabolized primarily in the liver (Keene and Jandl, 1965; Murray *et al.*, 1961). If free Hb is injected into the bloodstream, it is rapidly complexed by haptoglobin, and the complex is cleared from circulation, thus temporarily depleting the plasma of (the complexed) Hp (Noyes and Garby, 1967; Laurell and Nyman, 1957). In order to observe hemoglobinuria, the level of free Hb in circulation must exceed the level of Hp available for complex formation (Laurell and Nyman, 1957). Hemoglobinuric patients are found to have little or no plasma haptoglobin (Allison and ap Rees, 1957). Free Hb may get into the circulation in significant amounts as a result of hemolytic episodes. If an amount of free Hb sufficient to bind all the Hp is injected into circulation, Hp levels fall to undetectable levels within about 24 hrs (Laurell and Nyman, 1957). Normal Hp levels will return within a few days following total depletion. Haptoglobin is synthesized to the extent of about 30–50% of the intravascular pool each day (Noyes and Garby, 1967). There is no evidence that haptoglobin depletion causes an increase in the rate of Hp synthesis by a "feedback" mechanism. The effectiveness of haptoglobin in conserving hemoglobin (and, thus, iron) has been questioned on several accounts (Giblett, 1969), and the physiological role of haptoglobin is undoubtedly more complicated (Pintera, 1971).

Recently, Prof. Dr. Prokop and his collaborators have observed that human haptoglobins can behave as antibodies against streptococci possessing the T4 antigen. This effect varies according to the Hp phenotype, type 2-1 and 2-2 Hp's behaving as high titered complete antibodies, while 1-1 derived Hp acts like a "blocking" antibody (Köhler *et al.*, 1978; Prokop and Köhler, 1979; Prokop, 1979). Homologous animal haptoglobins exhibit the effect as well. These observations are particularly interesting in view of the structural homology between the short polypeptide chains of Hp and the light chains of the immunoglobulins (see below in 40.3.6).

40.3 Genetics and Biochemistry of Haptoglobins

40.3.1 Genetic variation in haptoglobin

Haptoglobin was well studied electrophoretically, both as free Hp and complexed with Hb, before the polymorphism

was fully recognized (Wieme, 1953; Jayle *et al.*, 1952; Tuttle, 1955a and 1955b). In 1947, Jayle and Gillard noticed that there appeared to be more than one kind of haptoglobin present in plasma on the basis of ammonium sulfate fractionation, which was part of the Hp purification procedure. In 1955, Smithies found the genetically controlled variation in haptoglobin by starch gel electrophoresis. These studies were the same ones in which the now familiar zone electrophoresis technique on starch gels was elaborated (section 2.3.4). Three different starch gel electrophoretic patterns of serum proteins could be observed, the differences lying in those proteins between the so-called "fast" and "slow" α_2 globulins. Complex changes occurred in the banding patterns depending upon the amount of Hb present. The proteins all bound hemoglobin, and were easier to type in its presence (Smithies, 1955a). The different "groups" were originally called I, II_A and II_B, and were well distributed even in a small sample of sera from different individuals. It was suggested (Smithies, 1955b) that these differences had a genetic basis. Studies on eighteen families were consistent with a straightforward genetic model in which "group I" and "group II_B" were homozygotes for each of a pair of codominant alleles, and "group II_A" was the heterozygote (Smithies and Walker, 1955). It was quickly realized that the proteins exhibiting this variation were identical to the hemoglobin-binding proteins of Polonovski and Jayle (1938), Wieme (1953), Tuttle (1955a), and others, namely haptoglobin (Smithies and Walker, 1956). Accordingly, it was suggested that this family of proteins be designated the "haptoglobin system", that the genetic locus be called *Hp*, and the two alleles Hp^1 and Hp^2. "Group I" was Hp 1-1 (Hp^1Hp^1), "II_A" was Hp 2-1 (Hp^2Hp^1) and "II_B" was Hp 2-2 (Hp^2Hp^2). These features of this serum group system were quickly confirmed (Moretti *et al.*, 1957; Sutton *et al.*, 1956), and the proposed mode of inheritance has been widely confirmed by family and population studies (e.g. Galatius-Jensen, 1956, 1958a and 1960; Prokop *et al.*, 1961; Kirk, 1968a and 1971). It was also clear that there were significant differences between the Hp allele frequencies in ethnically distinct populations (Sutton *et al.*, 1956; Allison *et al.*, 1958; Giblett, 1959), and that some Black African populations presented certain complexities in their Hp, not seen up to that time in Europeans (Allison *et al.*, 1958; Giblett, 1959).

40.3.2 Additional genetic variation at the Hp^1 locus—Haptoglobin "subtypes"

In 1962, Connell *et al.* subjected partially purified haptoglobins, representing the three common (1-1, 2-1 and 2-2) phenotypes, to electrophoresis after reductive cleavage in the presence of mercaptoethanol. Two classes of products were obtained, one of which appeared to be common to all Hp molecules, and which did not migrate in starch gels at acidic pH unless 8M urea was present. However, the other products migrated differently according to the phenotype. This latter, called the "hp 1" product, showed two different patterns, fast (F) and slow (S). This behavior was attributed to the presence of two Hp^1 alleles, Hp^{1F} and Hp^{1S}. With three alleles, Hp^{1F}, Hp^{1S} and Hp^2, six phenotypes could be observed (provided appropriate electrophoretic conditions were employed): Hp 2-2, Hp 2-1F, Hp 2-1S, Hp 1F-1F, Hp 1F-1S and Hp 1S-1S. Family studies by Smithies *et al.* (1962a) indicated that this genetic model was correct, and a survey of a small number of Europeans (from Toronto and Madison, WI) indicated that the frequencies of Hp^{1F} and Hp^{1S} were about 0.16 and 0.24, respectively, assuming Hp^2 to be about 0.6. This genetic hypothesis has been confirmed (Shim and Bearn, 1964a; Ehnholm, 1969; Fagerhol and Jacobsen, 1969). Fagerhol and Jacobsen (1969) used a different discontinuous buffer system for the resolution of the Hp 1 subtypes, and originally called them "E", "K" and "R", but said that they were equivalent to Hp 1F-1F, 1F-1S and 1S-1S, respectively. Electrophoretic patterns of the three common Hp phenotyhpes as seen on starch gels at pH 8.6, and those of the α polypeptides in the subtypes under denaturing conditions, are shown in Figure 40.1. The biochemical genetics of haptoglobin types and subtypes is discussed further below.

40.3.3 Other Hp variants and Hp 0

A number of variants of haptoglobin have been found, and the genetic explanation for some of them is not as clear as it is for others. The variants are described in this section. The genetic basis for their occurrence is considered later in connection with the structure and biosynthesis of the haptoglobins. Giblett (1969) usefully classified the Hp variant phenotypes as "quantitative" or "qualitative".

40.3.3.1 Quantitative variants. The majority of quantitative variants are modifications of the 2-1 phenotype. The Hp 2-1(mod) (Giblett, 1959), or Hp 2-1M (Connell and Smithies, 1959) phenotype shows a spectrum of patterns, ranging from a high concentration of the slower moving polymers (see below) with no visible fast Hp 1 band, to a high concentration of the fast band with only one of the members of the polymeric series visible (Giblett, 1969). Hp 2-1M (where "M" and "mod" indicate "modified") occurs at appreciable frequencies in the Black population (some 10% of American Blacks), but only occasionally in Europeans. Harris *et al.* (1960) described Hp 2-1M in a White family, and said that the pedigree indicated either a Hp^{2M} gene at the *Hp* locus, or modifier gene at another locus. If the Hp^{2M} gene was present, it gave a Hp 2-1M pattern in Hp^1Hp^{2M} people, but a 2-2 pattern indistinguishable from the usual one in Hp^2Hp^{2M} people. Giblett and Steinberg (1960) examined about 500 sera from American Blacks in 92 families, and found about 15% of a sample of unrelated individuals to be Hp 2-1M. They thought that the phenotype was a manifestation of an Hp^{2M} allele. Sutton and Karp (1964) thought that Hp 2-1M could be divided into four classes, called b, c, d and e, on the basis of the shift toward the faster moving bands. They believed that there were two alleles, Hp^{2cd} and Hp^{2e}, controlling the 2-1M phenotypes. Parker and Bearn (1963) offered an alternative explanation based on variation at a regulator gene locus,

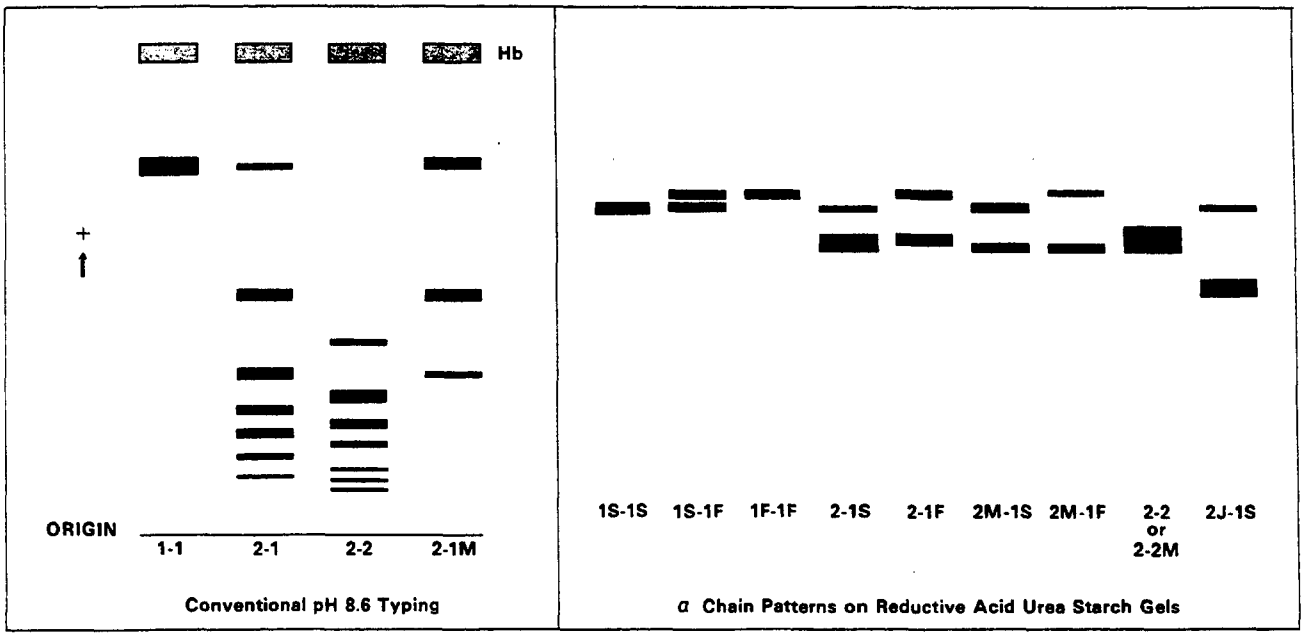


Figure 40.1 Electrophoretic Patterns of Hp Types and Subtypes

close to but not identical with the *Hp* locus. The genetic basis for Hp 2-1M is apparently still not completely understood.

Galatius-Jensen (1958b) found a phenotype which represented quantitative variability in the Hp^1 product. It was called 'Hp Carlberg', usually written Hp Ca. It has been observed in other populations as well (Giblett, 1964; Nance and Smithies, 1964; Harris *et al.*, 1959). The electrophoretic pattern resembles that of a mixture of Hp 2-2 and 2-1 in variable proportions. Subtyping shows reduced Hp^1 product in relation to Hp^2 product, although the ratio can vary even within the same family. Sutton (1965) has suggested that Hp Ca may be the result of genetic mosaicism, different cell populations in the same person producing Hp 2-2 and Hp 2-1. Hp Ca is inherited, however, and if mosaicism is the explanation, then an inherited tendency toward cellular somatic mosaicism would have to be postulated (Giblett, 1969).

Two other variants, described by Giblett (1964), are Hp 2-1(trans) and Hp 2-1(Haw). The 2-1(trans) exhibits a pattern that shows an increase in the faster moving components and a proportionate decrease in the slower ones. This shift is not as pronounced as in Hp 2-1M, and gives the appearance of a 'transitional' phase in going from an Hp 2-1 to an Hp 2-1M pattern (hence, the name 'trans'). Hp 2-1 (Haw) was so named because it was found in the serum of an Hawaiian subject. The pattern is similar to 2-1M in having a heavy concentration of the fastest bands, but differs in that there is no associated increase in the intensity of the second band nor a relative decrease in the intensities of the third and fourth bands.

One of the more puzzling kinds of quantitative variation is represented by the phenotype "Hp 0", which denotes anhaptoglobinemia or hypohaptoglobinemia. Failure to detect Hp by most of the electrophoretic methods used does not necessarily mean that it is completely absent; it usually means that the amount of Hp present is less than 15–20 mg/100 mℓ expressed as Hb-binding capacity (Giblett, 1969).

In 1958, Allison *et al.* tested 99 Nigerian sera for Hp and found that a little over 32% of them had no detectable activity. The finding was further discussed by Allison in 1958. Sutton *et al.* (1956) saw no Hp in a small sample of bloods from the Ivory Coast. Giblett (1959) found that about 4% of about 400 Black people from Seattle were Hp 0. Hp 0 is much more common as the result of matings involving an Hp 2-1M or Hp 0 parent, and it is rare if the parents are Hp 1-1 (Giblett and Steinberg, 1960; Sutton and Karp, 1964). The $Hp^{2M}Hp^2$ genotype, when expressed, cannot be distinguished from Hp^2Hp^2. The offspring of Hp 2-1M × 2-1M matings are found to be Hp 1-1, Hp 2-1M or Hp 0. It appears that $Hp^{2M}Hp^{2M}$ people are usually (or always) Hp 0. Black people who are phenotypically Hp 0 are often $Hp^{2M}Hp^1$ or $Hp^{2M}Hp^2$ (Giblett and Steinberg, 1960), although they can have apparently normal genotypes (Hp^2Hp^2 or Hp^2Hp^1) as well. These genotypical interpretations were given on the assumption, of course, that there really is an Hp^{2M} allele. Parker and Bearn (1963) proposed that Black populations carried a mutant in a regulator gene, and that Hp 0 could represent homozygosity for this mutant allele. There is evidence that such a gene may occur in Caucasian populations as well, albeit at very low frequencies (Murray *et al.*, 1966). In spite of the difficulties associated

with proving regulator gene hypotheses, they do appear more attractive than the original one based solely on Hp^{2M}. It is quite likely that the factors involved in determining Hp 2-1M and Hp Ca are applicable to Hp 0. There is some evidence for the existence of a very rare Hp^0 allele (Harris *et al.*, 1958a; Matsunaga, 1962; Schwerd and Sander, 1967). In these families, a homozygous Hp 1-1 or 2-2 parent had one or more children who appeared to be homozygous for the other allele. In some cases, Hp^0Hp^1 and Hp^0Hp^2 combinations are expressed like Hp^1Hp^1 or Hp^2Hp^2, respectively, but in other cases the phenotype is Hp 0.

Giblett (1969) noted that, in view of the fact that an Hp 1 phenotype could be the result of Hp^1Hp^1 or Hp^1Hp^0 genotypes, and similarly for the Hp 2 phenotype, the phenotypic nomenclature should reflect this fact. For some reason, homozygous haptoglobin types are more often expressed as "1-1" or "2-2", rather than just "1" or "2". For most other systems, phenotypic nomenclature denotes what is observed rather than implying a genotype. Giblett said that this latter practice should be followed with haptoglobin as well, and the suggestion is a good one. In summary, it appears that there is more than one genetic explanation for Hp 0, and that the one based on an operator or regulator gene mutation is not completely developed as yet.

It is important to note, before leaving the subject of anhaptoglobinemia, that haptoglobin genotypes are not fully expressed in a majority of fetuses and newborns. Many are, therefore, "anhaptoglobinemic", but this condition is transitory and reflects the rate at which the *Hp* genes become active. Detectable haptoglobin is usually found in about 10-15% of cord blood and newborn sera (Rausen *et al.*, 1961), but this percentage increases rapidly until it reaches nearly adult levels by about 4 months age (Hauge *et al.*, 1970). Few infants have less than 30 mg/100 mℓ haptoglobin at 6 months age (Bergstrand *et al.*, 1961). The Hp type expressed in cord blood and newborn serum reflects the genotype of the infant, and not that of the mother (Hirschfeld and Lunell, 1962; Siniscalco *et al.*, 1963). Hirschfeld and Lunell (1962) found an Hp 2-1 mother with a pair of twins (who had been stillborn), and the twins had Hp 1 and Hp 2 phenotypes. The data of Siniscalco *et al.* (1963) suggested that the onset of Hp synthesis in infants might depend on the maternal genotype, while Hauge *et al.* (1970) noted that the Hp 2 phenotype tended to develop more slowly than the others.

40.3.3.2 Qualitative variants. These variants contain components which are not present in the common phenotypes, and they are all very rare. They may be further classified as α chain variants or β chain variants, since it is now clear that the two kinds of polypeptide chains are coded for by different and independent genetic loci (see below). The best known rare variant is Hp 'Johnson', originally observed in a Black woman and her daughter in Seattle by Dr. Giblett. It reveals one or the other of the 1S or 1F α peptides, and a much slower, heavily-staining α chain (Smithies *et al.*, 1962a; Giblett and Brooks, 1963). The polypeptide made by the 'Johnson' allele was originally called hp 2Jα, but Giblett (1969) suggested that it should perhaps be called 'α^J' or 'α^3' (the latter, since it may be the result of a partial gene triplication—see in 40.3.6). When Hp^J is heterozygous with Hp^2, the haptoglobin synthesis is greatly reduced, and it is only possible in the occasional specimen to determine the electrophoretic pattern (Giblett, 1969). Isolated examples of Hp 1-J have been seen in such widely disparate populations as American Blacks, Kurdish Jewish, Australian aborigine and European (Smithies *et al.*, 1962b). Mukherjee and Das (1970) described a 2-1J in a Bengali Hindu, and Höglund *et al.* (1970) found 7 Hp 1-J types among 15,601 Swedish adults. Minor differences in the patterns are consistent with independent origin of the genes according to the unequal crossing over hypothesis (see below), although the samples were not freshly drawn when compared. In 1966, Giblett *et al.* described two more structural variants, which were the result of a new allele Hp^B. Both 1-B and 2-B phenotypes were observed. The α polypeptide conditioned by Hp^B migrates between hp α^2 and hp α^{1S}. Hp 1-B and 2-B are referred to as the "Ba" types by Giblett (1969). It is possible too, she said, that these represent β chain variations. Renwick and Marshall (1966) described the Hp 2-1D phenotype, thought to be the result of an allele Hp^{1D} paired with Hp^2. In the presence of saturating amounts of Hb (which is the way Hp is usually typed), Hp 2-1D and Hp 2-1 are not distinguishable. But under "subtyping" conditions, a band which runs faster than 1F is revealed (representing the α^{1D} peptide). The "D" stood for "dashing" in the name.

The so-called 'Marburg' phenotypes were originally seen in a German family (Aly *et al.*, 1962), and have been extensively studied by Cleve and Deicher (1965) and Weerts *et al.* (1965). All the electrophoretic components of Hp 2-1Mb demonstrated atypical immunological reactions with certain anti-Hp sera. The antigenic determinant called "B" (see below), which is on the β chain and is normally blocked in the Hp-Hb complex, still reacted even when saturating amounts of hemoglobin were present. Subtyping revealed no unusual α polypeptide chains, and it was concluded that this variant represented a β chain mutation. Bowman and Cleve (1967) have shown that the 'fingerprint' of the β chain of Hp Mb is different from the usual one. Another phenotype, called Hp 2-1 Bellevue, was described by Javid (1967a). It had immunological properties resembling those of the 'Marburg' haptoglobins, but the electrophoretic pattern differed. Electrophoresis under subtyping conditions suggested that the phenotype represented heterozygosity for a β chain variant. The 35 year old Black propositus had three sons who had apparently inherited the variant gene.

Other variants have been described which may represent β chain mutations. In 1964, Robson *et al.* described five new phenotypes called Hp 1-P, 2-P, 1-H, 2-H and 2-L. The electrophoretic behavior of the components of these haptoglobins differed in the presence and absence of hemoglobin, suggesting that something was unusual about their ability to complex with Hb. The alleles thought to be responsible for these types were called Hp^P, Hp^H and Hp^L. A similar

variant was reported by Giblett (1964). Hp Ab, as it was called, was found in a woman in Boston by Dr. Irving Umansky. The α polypeptides in these phenotypes migrated like 1F, 1S and 2 α chains usually do. The low pH of the gels used for subtyping might prevent separation of the variant chain (if it were there) by protonating carboxyl groups (Nance and Smithies, 1964). Amino acid residues which were neutral or equally charged could also be involved. Shim *et al.* (1965) said that the P and L variants might be additional mutants at the β chain locus.

The structure of haptoglobin is discussed below, but the molecule is known to consist of α and β chains, coded for by different loci. The so-called *Hp* locus is the α chain locus, and most of the variants, including the three common types representing the polymorphism, are α chain variations. Some β chain variants are now known, however, and it would be useful to distinguish between the structural loci in the nomenclature. Javid (1967a) showed that the α and β loci are not closely linked, and suggested Bp as a symbol for the β chain locus. The common allele would be Bp^A and Bp^B would represent the allele seen in Hp 2-1 Bellevue. Giblett (1969) suggested, however, that it would be preferable to call the α chain locus Hp_α and the β chain locus Hp_β, and this seems like the best idea. The electrophoretic patterns exhibited by some of the Hp variants are shown in Figure 40.2. A useful table of many of the variants was given by Kirk (1968a), and is reproduced in Pintera (1971) and in Putnam (1975c).

40.3.4 Structure of the haptoglobins

Efforts to purify and characterize haptoglobin have been undertaken by many workers, since the first work by Jayle and his collaborators as summarized by Jayle and Moretti (1962). One of the first purification schemes was devised by Jayle and Boussier (1954). Refinements were developed by Guinand *et al.* (1956), Jayle *et al.* (1956), Moretti *et al.* (1958) and Herman-Boussier *et al.* (1960). Haptoglobin has been isolated from serum, from ascitic fluid in some patients, and from the urine of certain patients who excrete it if it is of the 1-1 type. Purification methods for haptoglobin are discussed in some detail in Pintera (1971) and in Putnam (1975c).

The monomeric unit of human haptoglobin (represented by the molecule found in type 1-1 people) is composed of four polypeptide chains, two α chains and two β chains, connected by disulfide bridges (Shim and Bearn, 1964b; Malachy and Dixon, 1973a and 1973b; Malachy *et al.*, 1973). The general structure of the molecule (Figure 40.3) is reminiscent of the structure of the IgG molecule (Figure 1.41). The chain can be dissociated by reductive cleavage with mercaptoethanol in the presence of 8M urea (Smithies *et al.*, 1966; Connell *et al.*, 1966). The Hp 1-1 molecule is the only one, from among the three common types, that appears to be homogeneous. It migrates as a single band in starch gel electrophoresis, whether complexed with Hb or not, and exhibits a single ultracentrifuge peak. The 2-1 and 2-2 types contain more than one protein species, which can be observed in the ultracentrifuge, or by starch gel electrophoresis (Bearn and Franklin, 1958 and 1959), and which differ to some extent immunologically (Korngold, 1963; Eichmann *et al.*, 1966). The multiplicity of bands seen upon starch gels with 2-1 and 2-2 types is familiar to anyone who has typed haptoglobin. These bands are now known to represent a series of polymers, which are, however, very stable,

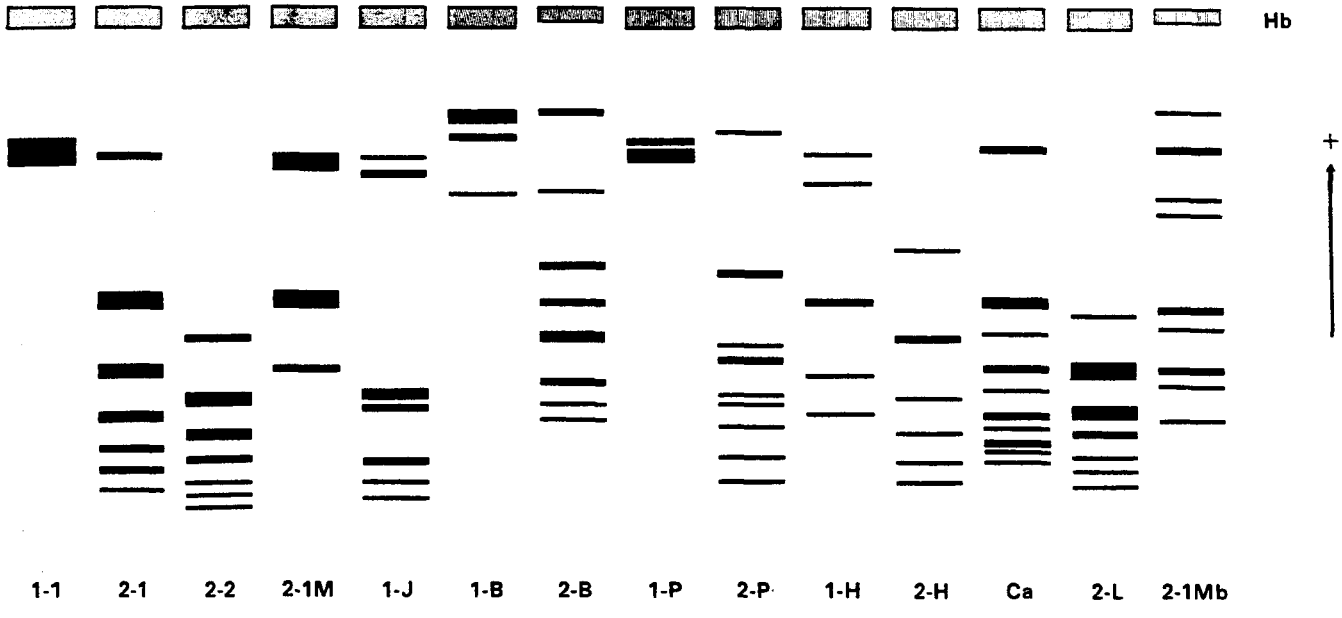


Figure 40.2 Electrophoretic Patterns of Some Haptoglobin Variants

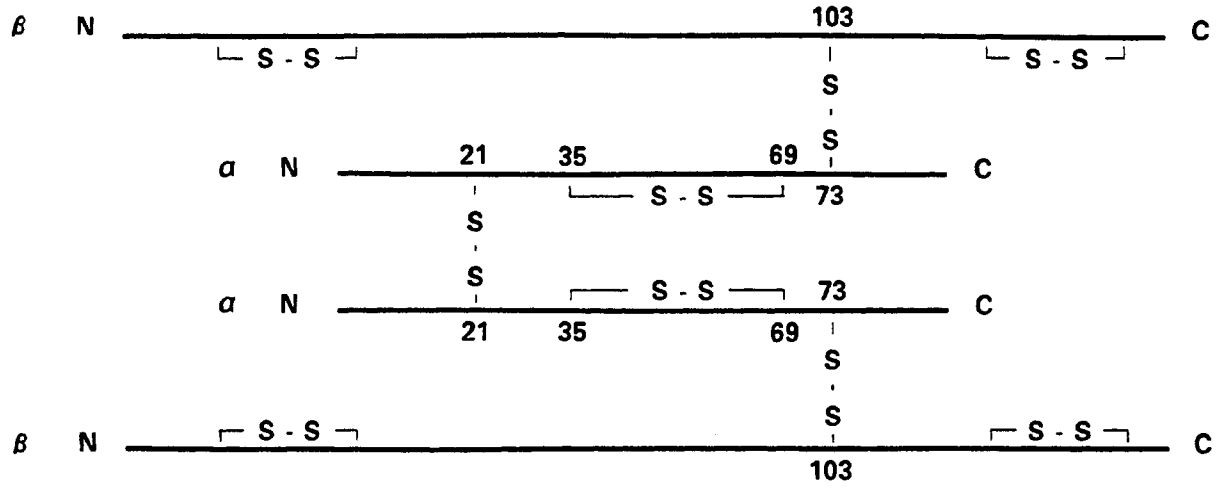


Figure 40.3 Diagrammatic Structure of Monomeric Haptoglobin

and not artifacts (Connell and Smithies, 1959). The characteristic 2-1 pattern cannot be produced by the mixing of 1-1 and 2-2 sera. The nature of the individual components in the 2-2 polymer series turned out to be rather complex, and was finally worked out by Fuller *et al.* in 1973. Until that time, there was disagreement as to the number of α and β chains in the polymers, and whether each successively larger member of the series represented the addition of a β chain, a half molecule (essentially $\alpha\beta$) or an entire monomer. Fuller *et al.* (1973) isolated several discrete polymers, and could show that each consisted of α^2 (the α chain coded for by the Hp^2 allele) and β chains in a 1:1 ratio. Members of the series differed from one another by an average MW increment of 54,500 daltons, approximately equivalent to one α^2 chain (17,300) and one β chain (40,000). Reductive cleavage experiments established that the "$\alpha^2\beta$" units were joined by disulfide bridges to form the polymers, and the polymers corresponded to a series $\alpha_n\beta_n$, n = 3 to 8. Javid (1964) carried out experiments on the differences between the 2-1 and 2-2 patterns, confirming the thinking of Allison (1959) and Parker and Bearn (1963) that α^1 chains (the α chain product of the Hp^1 allele) are incorporated into the polymers formed by the α chain products of Hp^2 (α^2 is the higher MW chain, and is responsible for the polymerization phenomenon) in the 2-1 type. The Hp 1-1 molecule is thus $(\alpha^1\beta)_2$ while the 2-2 haptoglobins consist of a series of molecules $(\alpha^2\beta)_n$, n = 1,2,3. . . . . Type 2-1 haptoglobins are expected to be a series of polymers of the kind $(\alpha^1\beta)_m$—$(\alpha^2\beta)_n$.

The nature of the Hp·Hb complex has been widely studied using a variety of methods, and these are reviewed by Putnam (1975c). The exact nature of the binding has not been completely worked out, and this is still an active area of investigation. Haptoglobin combines stoichiometrically with hemoglobin A to form a very stable complex, the binding being so tight that the complex formation reaction is considered irreversible. The heme moiety of Hb is unimportant in the linkage, though Hp combines with oxyhemoglobin, and not with deoxyhemoglobin. The oxygen equilibrium of the complexed Hb is greatly altered (Nagel and Gibson, 1971). The combination is not species-specific, and human Hp can combine with animal hemoglobins. Hp can likewise combine with Hb F and with some of the abnormal hemoglobins (see in section 38). Bearn and Franklin (1958) complexed Hp with Hb C in order to cause the complex to have a slower electrohoretic mobility than it would have had with Hb A. The α chains of Hb are essential for binding to haptoglobin, though they bind more weakly than intact Hb, but much more strongly than isolated Hb β chains. Human Hb α chains will bind animal haptoglobins, and Terpstra and Smith (1976) have, for example, studied Hb α chain binding to porcine Hp. There is recent evidence (Kazim and Atassi, 1980), however, that Hp may in fact bind Hb β chains quite strongly under certain conditions, but that special assay techniques are required to demonstrate the interaction. Laurell (1960) suggested that the binding of Hp to Hb occurs through the $\alpha\beta$ dimer of hemoglobin. The fully saturated Hp 1-1 (the simplest haptoglobin) is, in this view, bound to two halves of an Hb molecule rather than to one intact molecule, and there is considerable experimental evidence that this view is correct. The data of Nagel and Gibson (1971) suggested that Hp possesses four binding sites, two for each $\alpha\beta$ Hb dimer. One pair of sites in Hp binds a Hb $\alpha\beta$ dimer, and thereby induces an allosteric change in Hp creating a second site for an Hb $\alpha\beta$ dimer. The exact nature of the binding regions in the two molecules, and of the amino acids actually involved, is not completely known. Most of the evidence is indirect, and based upon various molecular probe studies, e.g. Russo and Chen (1976), Osada *et al.* (1978), Katnik and Dobryszycka (1978) and Hwang and Greer (1979). Conformational changes in Hp are clearly

involved, and Hevér (1977) has shown that the reduction of Hb binding capacity by the haptoglobin in heated serum varies with Hp type and subtype.

40.3.5 Subunit and polypeptide chain structure

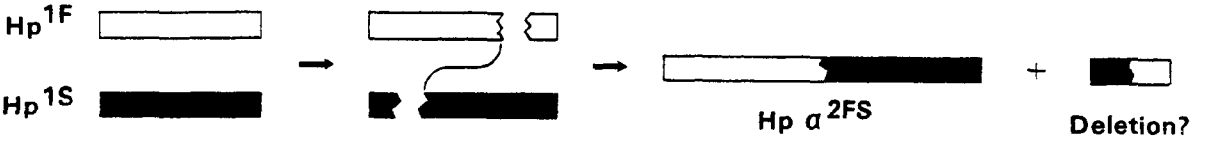
Haptoglobin resembles the immunoglobulins in subunit structure, as noted above. There are two α (light) chains and two β (heavy) chains (Fig. 40.3). There are a number of different α chains, because it is the α chain structural locus which exhibits most of the genetic variation except for a few rare variants of the β chain. The three major Hp types can be divided into six subtypes on the basis of the electrophoretic behavior of their polypeptide chains following reductive cleavage in urea (Connell *et al.*, 1962). Electrophoresis under these conditions reveals that there are only two polypeptide chains in Hp, α and β, in spite of the multiplicity of bands seen in starch gels at pH 8.6 (explained above). Only the α chain patterns are of interest in electrophoretic subtyping (Fig. 40.1), since all six types have common β chains (Cleve *et al.*, 1967) which migrate very slowly in acid-urea starch gels. It has become more or less conventional to designate haptoglobins with the symbol "Hp", the polypeptide chains as "hp", and the genes as *Hp*. The α chain structural locus should really be designated Hp_α, as suggested by Giblett (1969), to distinguish it from Hp_β, now that β chain variants have been found. The α chains have been designated in a number of different ways by various workers, and these usages can be quite confusing to non-haptoglobin specialists. There are, essentially, three kinds of α chains, produced by the $Hp_\alpha{}^{1S}$, $Hp_\alpha{}^{1F}$ and $Hp_\alpha{}^{2}$ genes. The $Hp_\alpha{}^{1S}$ locus produces hp α^{1S}, which has also been called "hp 1Sα"; similarly, $Hp_\alpha{}^{1F}$ produces α^{1F}, and $Hp_\alpha{}^{2}$ produces α^2, which have also been designated in the alternative ways shown for α^{1S}.

The classical Hp 1-1 phenotype can have haptoglobin with hp α^{1F} chains, with α^{1S} chains, or with equal quantities of both. The subtype phenotypes (and genotypes) are then, respectively, Hp 1F-1F ($Hp^{1F}Hp^{1F}$), Hp 1S-1S ($Hp^{1S}Hp^{1S}$) and Hp 1F-1S ($Hp^{1F}Hp^{1S}$). The classical 2-1 phenotype may be subtyped into Hp 2-1F or Hp 2-1S, which contain hp α^2, and either hp α^{1F} or hp α^{1S} chains, but not both. Hp 2-2 contains hp α^2 chains (although there may well be molecular variants of α^2 chains—see further below).

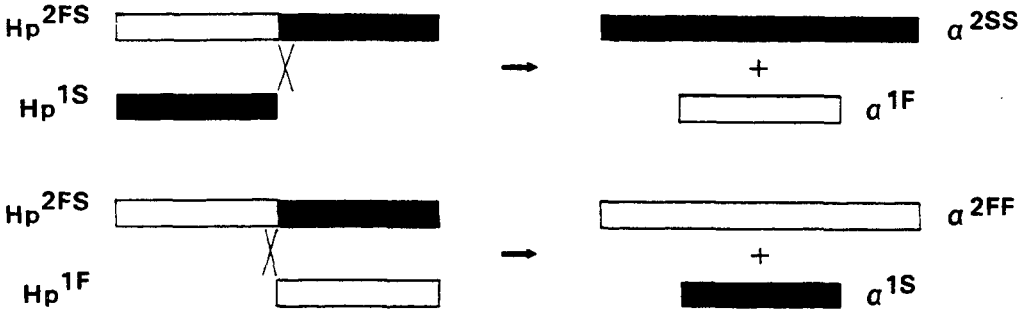
The primary structure of the Hp constituent polypeptide chains has been determined, and the α chain sequences have been most informative from a genetic point of view. The sequence work on the α chains may be found in the papers of Black and Dixon (1968 and 1970), Black *et al.* (1970), Malachy and Dixon (1973a and 1973b) and Malachy *et al.* (1973). The α^{1S} and α^{1F} chains contain 84 amino acid residues, and are identical except that α^{1S} has Glu at position 54 where α^{1F} has Lys. The α^2 chain is almost twice as long as the α^1 chains, containing 143 amino acid residues. But what is most extraordinary about the hp α^2 chain sequence is that it consists of the first 71 and the last 72 amino acid residues of hp α^1, joined together to form a 143 residue chain. The Asp13-Ala71 of α^1 is repeated in hp α^2 as Asp72-Ala130. One of the repeated sequences is from α^{1S} and the other is from α^{1F} (which can be discerned because of the Glu/Lys difference mentioned above). The hp α^2 polypeptide chain represents the first example of a partial gene duplication fully documented by amino acid sequence analysis. The point is discussed more fully in the following section. The hp α chains contain Cys at α21 and form an α21–α21 inter-α chain disulfide bridge. There is an α35–α69 intra-α chain disulfide linkage, and the Cys forming the α-chain half of the interchain disulfide bridge to the β chain is at α73. The sequence of the β chain has been almost completely worked out (Barnett *et al.*, 1972; Kurosky *et al.*, 1976), and the α chain is attached to it through β103 Cys. All the carbohydrate in haptoglobin appears to be associated with the β chain, and is probably attached (at least in part) through β23 Asn (Kurosky *et al.*, 1976).

40.3.6 Biochemical genetics

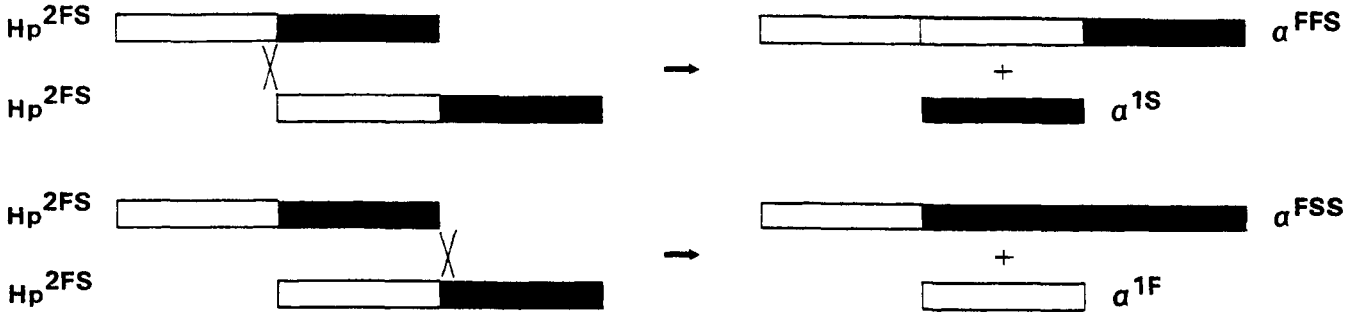
Soon after the observation of the haptoglobin polymorphism, it became clear that the monomeric molecule was of the $\alpha_2\beta_2$ structure, and that the α chain locus was responsible for the genetic variation. Early peptide analyses of the α^{1S}, α^{1F} and α^2 chains indicated considerable similarity between α^{1S} and α^{1F}, but a great difference in size between them and the α^2. The peptide analysis suggested to Smithies *et al.* (1962b) that the α^2 chain was almost a duplicate of the α^1 chains, and that α^2 had arisen because of a partial gene duplication of the α^1 genes caused by a homologous but unequal crossover at the Hp_α locus. This event gives rise to an α chain which contains most of α^{1F} and most of α^{1S} in the same polypeptide chain, namely α^2, and for this reason the α^2 chain can be designated α^{2FS}. The way in which the partial gene duplication may have occurred is shown in Figure 40.4A. During meiosis in an Hp 2-1 individual, when homologous chromosomes pair with one another, the α^1 gene on the one chromosome (whether it is α^{1S} or α^{1F}) will find itself unable to pair with the almost twice as long α^2 gene; however, because of the structural homology, the α^1 can readily pair with one or the other half of α^2 (the duplicated half). Therefore, as Smithies (1964) discussed in detail, once the partial gene duplication has occurred, the possibility for further genetic variation is increased. Because perfect synapsis is impossible between either α^1 gene and the α^{2FS} gene in a 2-1 individual, the α^{1S} or α^{1F} genes will pair with a segment of the α^{2FS} gene, as shown in Fig. 40.4B. Crossovers in such heterozygous synapses can then lead to different α^2 chains, namely α^{2FF}, α^{2SS} and α^{2SF}. In a similar way, because of the duplicative chain homology, displaced synapsing could occur in homozygous 2-2 individuals (Fig. 40.4C). Here, part of one α^{2FS} gene pairs with an (almost) homologous part of the other α^{2FS} gene (the "F" and "S" parts differ in a single amino acid). A crossover under these circumstances could lead to two varieties of triplicated genes, giving α^{FFS} or α^{FSS} chains. It is believed that Hp Johnson contains just such a chain, and these are thought to have arisen independently in the various different populations in which they have been



A. Possible Mechanism for Formation of the Hp^{2}_{α} Gene



B. Unequal Crossing Over in Hp 2-1 Heterozygotes



C. Mechanism of Partial Triplication by Displaced Synapse in Hp 2-2 Homozygotes

Figure 40.4 Diagrammatic Representation of Possible Unequal Crossovers in Hp α Chains (modified from those in Giblett, 1968 and 1969).

observed. Such genetic events are not expected to be common, particularly in the Hp 2-2 case, where completely homologous pairing is possible, and it is not particularly surprising, therefore, that 'Johnson' phenotypes are quite rare. Giblett (1968 and 1969) has suggested that if the triplication hypothesis is confirmed for Hp 'Johnson', then the resulting α chain should be called α^3. Nance and Smithies (1963) subtyped many sera and found α chains in some with migration rates that could well correspond to α^{2FF} or α^{2SS}. These genes are apparently infrequent, however (Shim and Bearn, 1964a). While the nature of many of these "probable" Hp α chains will eventually be determined by sequence analysis, there are considerable technical difficulties associated with telling the difference between chains like α^{FS} and α^{SF}, for example. Haptoglobin biochemical genetics is discussed in Giblett (1969), Harris (1975) and Putnam (1975c).

Another point of some interest is the structural homology between the Hp polypeptide chains and those of other proteins. The α chains bear structural homology to the light (κ or λ) chains of IgG. On this basis, it was expected that the Hp β chain would show structural homology with the IgG heavy chains, but this expectation has not been fulfilled. Instead, an unexpected homology was found between Hp β and the corresponding regions of the so-called chymotrypsin family of serine proteases. This matter is further discussed by Putnam (1975c) and by Kurosky *et al.* (1976).

40.4 Medicolegal Applications

40.4.1 Disputed parentage

The Hp system is applied in cases of disputed paternity in many laboratories. The use of the system is discussed extensively by Galatius-Jensen (1956, 1958a, 1960 and 1962), Baitsch (1961), Giblett (1963) and Prokop and Bundschuh (1963). The probability of excluding a falsely accused father in Western European populations is about 18%, placing it among the better systems for this purpose. In the U.S. population, the chances of excluding a falsely accused father are about 18% for both White and Black people according to Chakraborty *et al.* (1974), and about 18% for Whites and 15% for Blacks according to Dykes and Polesky (1978). The system is not widely employed in U.S. laboratories, however (Polesky and Krause, 1977), but the percentage of laboratories using Hp routinely is higher among AABB Reference Laboratories than among other laboratories.

The most serious problems that could arise in the application of Hp to disputed paternity cases would involve the apparently very rare silent allele Hp^0. Many newborns and infants are temporarily anhaptoglobinemic, and their sera cannot be typed until they are a little bit older. In addition, there are occasional anhaptoglobinemic adults (section 40.3.3.1). These people would simply not give a typing result. There may be more than one genetic basis for anhaptoglobinemia in older children and adults, and the condition is comparatively more common in people of Black African origin than in Europeans. The Hp 2-1M phenotype, which is also more frequent in people of Black African origin, appears to be related in some way to the anhaptoglobinemia phenomenon, and could, if encountered, lead to difficulty in the interpretation of the inheritance patterns. Most laboratories utilize conventional Hp typing in these cases, under which circumstances the three common phenotypes can be detected. The discriminating power of the system would be increased by the application of Hp^1 subtyping.

40.4.2 Haptoglobin typing in dried bloodstains

A number of different methods have been used for the phenotyping of Hp in bloodstains. Early studies on the use of starch gel techniques similar to those used for serum typing were only partially successful (Dürwald, 1961 and 1963; Falk and Bundschuh, 1963), although some stains could be typed. Prokop and Bundschuh (1963) pointed out that the results to be expected from stains depended on the substratum upon which the blood is deposited because of the differences in extractability of the serum. Culliford (1963) obtained fair, but not completely satisfactory results with bloodstains using starch gels and the discontinuous buffer system of Poulik (1957).

In 1966, Culliford and Wraxall described an immunoelectrophoretic technique for Hp phenotyping in bloodstains which was found to be more reliable than starch gel procedures. Immunoelectrophoretic methods had been applied to Hp typing in serum by Hirschfeld (1959c) and by Fine and Battistini (1960). Hirschfeld (1968a) summarized the studies on the immunoelectrophoretic method. In this procedure, serum or bloodstain extract is electrophoresed in agar gel at about pH 8.6, after which a trough is cut in the gel and filled with an anti-Hp serum. After a suitable diffusion period, precipitin arcs develop, and those representing Hp 1, 2-1 and 2 phenotypes can be distinguished by their somewhat different positions (electrophoretic mobilities). Bargagna and Cave Bondi (1968) confirmed the usefulness of Culliford and Wraxall's (1966) procedure in bloodstain typing. They typed stains up to 69 days old. Whitehead and Morris (1969) presented a modification of the method which they said gave precipitin arcs that were easier to interpret in terms of phenotype. Katnik and Dobryszycka (1977) have carried out immunochemical studies on Hp in an effort to find out which amino acid residues in the molecule are involved in the antigenic determinants. The results indicated that tyrosyl residues are not essential. The immunochemical studies of Shim *et al.* (1965), in which anti-β and anti-α^2 chain sera were used, showed that the former reacts only with free Hp but not with Hp–Hb complex, while the latter reacted with either. The reason for the difference is that the β chain contains the Hb binding sites. The immunoelectrophoretic technique for bloodstain Hp typing is described in Culliford (1971) as well.

Electrophoresis is the preferred technique for Hp grouping. For whatever reasons, the starch gel methods that are usually used for serum typing are not very satisfactory for bloodstain typing (Dürwald, 1961 and 1963; Falk and Bundschuh, 1963; Culliford, 1963 and 1971; Turowska, 1969;

Hilgermann, 1972b). As a result, other electrophoretic support media have been employed for blood stains. Polyacrylamide gels appear to have proved most satisfactory. In 1971, Culliford described a reliable procedure for Hp typing in stains based on the use of polyacrylamide gel gradients. These were prepared in slab form using a gradient former, and the polyacrylamide concentration varied from about 5 to about 30%. More recently, gels of this kind have become commercially available. Earlier applications of horizontal nongradient polyacrylamide gel electrophoresis to Hp stain typing were not particularly successful (Gervais and Viescou, 1965), but Felix *et al.* (1977) reported satisfactory results using such a technique. Polyacrylamide disc gel electrophoresis has also been successfully used for bloodstain typing (Castilla *et al.*, 1972; Hilgermann, 1972a and 1972b). Hilgermann (1972b) could type stains up to 4 weeks old by polyacrylamide disc gel electrophoresis, and said that the technique was much better than starch gel or immunoelectrophoresis. The gradient gel procedure is fully described in MPFSL (1978). It has been used in connection with the Microzone electrophoresis system (Grunbaum, 1975), and Baxter and Rees (1974b) reported that Hp phenotypes and several Hb variants could be determined simultaneously in this way. Wraxall and Stolorow (1978) described a procedure using horizontal nongradient polyacrylamide gels which is fully satisfactory for typing bloodstains. Hp can be rapidly typed in serum samples on agarose gels (Rafowicz and Lavergne, 1974).

Haptoglobin is normally typed by electrophoretic separation of Hp–Hb complexes, and the separated proteins are detected through their peroxidase activity (which the Hb still possesses). Many of the reagents used for presumptive (catalytic) blood identification tests, based on the peroxidase activity of hemoglobin (section 6), have been employed for the detection of Hp–Hb complexes in gels following electrophoresis as well. Benzidine was often preferred until its use was discontinued because of its carcinogenicity. Owen *et al.* (1958) tested a series of oxidizable catalytic substrates, including guaiacol, leucomalachite green, amidopyridine, p-anisidine, benzidine, o-tolidine and o-dianisidine. They preferred o-dianisidine, which was also recommended by Compton *et al.* (1976). Queen and Peacock (1966) liked guaiacol; Burdett (1977) found leucomalachite green to be preferable in comparison with several noncarcinogenic compounds including phenolphthalin, diphenylamine, 2,6-dichlorophenol indophenol, N,N′-diethyl-p-phenylenediamine sulfate and 2,2′-diazo-di-(3-ethylbenzthiazoline-6-sulfonic acid). Singh (1967) recommended initial benzidine-peroxide staining, followed by an Amido Black 10B staining step to give clearer, more permanent bands.

Many investigators have noticed that older bloodstains become difficult to type for Hp. Until fairly recently, it was seldom possible to type bloodstains more than a few weeks to a few months old. Older stains tended to give streaky, overstained, and frequently unreadable patterns. One of the reasons apparently has to do with the fact that hemoglobin undergoes degradation in bloodstains, and the Hb degradation products are extracted from the stain along with the Hp. Various methods have been proposed to get around this difficulty. A related issue is the extractability of Hp itself in older bloodstains. It is common knowledge that older bloodstains do not yield up their constituent proteins to aqueous extraction media as readily as do fresher ones, and there is no reason for supposing that haptoglobin is an exception in this respect. Thus, one might fail to obtain readable typing results from a bloodstain for a variety of different reasons, such as (1) the sample could have been an Hp 0, or some other weakly expressed phenotype to begin with; (2) Sufficient Hp was not extracted to be detectable by the methods being used; (3) Degradation of the haptoglobin protein may have occurred; or (4) Hemoglobin degradation products may interfere with the electrophoretic typing. The first three of these problems could apply to any plasma protein or isoenzyme genetic marker. Studies by Nikolenko (1972 and 1975) in the U.S.S.R. and by Shaler *et al.* (1977) in this country indicate that procedures designed to better extract the Hp from bloodstains greatly improve the results with older specimens. Nikolenko's (1972 and 1975) results indicate that the addition of urea to the extraction buffers allows typing of somewhat older stains, presumably because haptoglobin is more efficiently extracted. The details of his work must be left to those with a better reading knowledge of Russian. Shaler *et al.* (1977) extracted bloodstains with buffer alone, and with buffers containing the detergents sodium dodecyl sulfate (SDS), Triton-X-100 and Tween-20, at pH's of 4.6 and 8.4. The quantity of Hp extracted was determined by "rocket electrophoresis" (section 2.4.3.1). At low pH, Triton and buffer extractions were about equally effective with stains of comparable age, while at higher pH, Triton and Tween were more effective. More important than the detergent effect, however, was the observation that the amount of immunologically detectable Hp extracted increased significantly (as much as 133%) with 24 hrs extraction as compared with 6 hrs. Extraction with detergent at low pH, and with buffer at high pH, was essentially complete at 24 hrs. Stains from 44 to 202 days old were tested with and without Triton in the extraction buffer, and the differences were not significant. Extraction at pH 8.4 was far superior to that at pH 4.6 with older stains, however. The common practice of soaking stains for a matter of minutes in electrophoretic gel buffer prior to electrophoresis is not, therefore, the best approach if one wants to type Hp in an older stain.

Improvements in bloodstain typing results have also been observed if the Hb and its apparent degradation products are separated from the stain extract prior to electrophoresis. Fresh Hb A must, of course, be added back to the sample before insertion into the typing gel. Gazaway (1976) separated the Hb material from the Hp by an agar gel electrophoresis procedure in cylindrical tubes. Conditions were employed in which the unwanted hemoglobin material migrated into the gel while Hp did not. A simpler procedure has recently been described by Stolorow and Wraxall (1979). The stain extract is subjected to a simple $CHCl_3$ extraction

in a test tube, and the aqueous layer removed for electrophoretic typing. Chloroform extraction is an old procedure for this purpose, dating back to the early studies of Formanek (1900) and of Krüger (1901). This procedure is applicable to the preparation of samples for electrophoresis by any desired typing method, and it is possible to type bloodstains some months older in this way than has routinely been possible.

A rather different approach involving specific immunoprecipitation of Hp from bloodstain extracts was reported by Blake and Sensabaugh (1978b). Subsequent typing in SDS-containing polyacylamide gels was possible in bloodstains up to 18 months old.

Haptoglobin cannot be detected in saliva or in semen (Blake, 1976; Blake and Sensabaugh, 1976 and 1978a; Schwerd and Fehrer, 1979). If the protein is present, the concentrations are extremely low, and the system is not applicable as a genetic marker in these fluids. In seminal plasma, Blake and Sensabaugh (1978a) could have detected Hp at concentrations of $\geqq 5.6\ \mu g/m\ell$, and they were unable to do so.

Chun and Sensabaugh (1979) reported preliminary but promising results on the possibility of subtyping Hp in blood stains. Immunoprecipitation and absorption procedures were employed for the isolation of the Hp from stain extracts. The discrimination index for the Hp system would be improved significantly if the Hp 1 subtypes could be discriminated.

Reviews of many aspects of the haptoglobin system may be found in Harris *et al.* (1959), Laurell (1960), Galatius-Jensen (1960 and 1962), Jayle and Moretti (1962), Giblett (1963, 1968 and 1969), Prokop and Bundschuh (1963), Javid (1967b), Kirk (1968a), Prokop and Uhlenbruck (1969), Pintera (1971), Braun (1972), Harris (1975) and Putnam (1975c).

40.5 Distribution of Hp Phenotypes in U.S. Populations

The data are shown in Table 40.1. The worldwide distribution of Hp phenotypes was discussed by Walter and Steegmüller (1969), and extensive tables may be found in Mourant *et al.* (1976). The highest Hp^1 frequency in major ethnic groups is found in Australian aborigines, and the lowest is found in Lapps. Walter and Steegmüller (1969) had data on almost 145,000 Caucasians. Hp^1 in European Caucasians was 0.386, and somewhat lower in those of non-European derivation (0.254). Hp 2-1M was seen at frequencies greater than 1% only in Negroes, but non-European Caucasians had a frequency of 0.5%. Hp 0 occurred in 4.5% of Negroes, and in 1–2% of non-European Caucasians, Mongoloids, Australian aborigines and Lapps. European Caucasians showed 0.5% Hp 0. Hp^{1S} seems to be more common than Hp^{1F} in most populations.

Table 40.1 Distribution of Hp Phenotypes in U.S. Populations

Population	Total	Frequency - Number (Percent) 1	2-1	2-1M	2	0	Note	Reference
CAUCASIAN								
Ann Arbor, MI	68	9(13.2)	40(58.8)		19(27.9)			Sutton et al., 1959
Michigan and Illinois	161	23(14.3)	77(47.8)		61(37.9)			Bayani-Siosin et al., 1962
Seattle, WA	409	54(13.2)	206(50.3)	0	149(36.4)		1	Giblett and Brooks, 1963
Southeastern GA	145	27(18.6)	64(44.1)	0	52(35.9)	2(1.4)		Cooper et al., 1963
Maryland	192	24(12.5)	85(44.3)		83(43.2)			Queen and Peacock, 1966
Chicago, IL	101	18(17.8)	51(50.5)		32(31.7)			Shih and Hsia, 1969
Tecumseh, MI	7,655	1,307(17.07)	3,734(48.78)	11(0.0014)	2,575(33.64)	22(0.0029)	2	Schreffler et al., 1971
Orange County, CA	185	(16)	(48)		(36)		3	Fitzpatrick et al., 1976
Bexar County, TX	200	(17)	(50)		(33)			Ganaway and Lux, 1978
Pittsburgh, PA	1,263	185(14.6)	555(43.9)	26(2.1)	495(39.2)	2(0.16)		Hagins et al., 1978
California	274	(14.6)	(49.3)		(36.1)			Grunbaum et al., 1978
Detroit, MI	503	69(13.72)	213(42.35)	0	220(43.74)		4	Stolorow et al., 1979
Miami, FL	366	58(15.8)	161(44)		141(38.5)	6(1.6)	4	Stuver, 1979
Los Angeles, CA	311	62(19.9)	149(47.9)		100(32.2)		4	Siglar, 1979
California	860	154(17.9)	418(48.6)		288(33.5)			Grunbaum et al., 1980
NEGRO								
Seattle, WA	406	(26.4)	(38.2)	(9.8)	(21.4)	(4.2)		Giblett, 1959
Ann Arbor, MI	48	17(35.4)	17(35.4)		9(18.8)	5(10.4)		Sutton et al., 1959
Seattle, WA and Cleveland OH	178	45(25.3)	70(39.3)	27(15.2)	31(17.4)	5(2.8)		Giblett and Steinberg, 1960
New York, NY	100	(40)	(38.9)	(0.08)	(21.1)	(0.02)		Parker and Bearn, 1961
Seattle, WA	1,657	472(28.5)	641(38.7)	181(10.9)	307(18.5)	56(3.4)	5	Giblett and Brooks, 1963
Southeastern GA	167	48(28.7)	61(36.5)	13(7.8)	42(25.1)	3(1.8)		Cooper et al., 1963
Chicago, IL	101	30(29.7)	47(46.5)		24(23.8)			Shih and Hsia, 1969
Bexar County, TX	200	(35)	(49)		(17)			Ganaway and Lux, 1978
Pittsburgh, PA	721	206(28.6)	293(40.6)	66(9.2)	148(20.5)	8(1.1)		Hagins et al., 1978
California	124	(33.9)	(41.9)		(21)		6	Grunbaum et al., 1978
Detroit, MI	504	142(28.17)	204(40.48)	59(11.71)	84(16.67)	11(2.18)	4,7	Stolorow et al., 1979
Miami, FL	346	120(34.7)	164(47.4)		40(11.6)	22(6.4)	4	Stuver, 1979
Los Angeles, CA	130	47(36.2)	58(44.6)		25(19.2)		4	Siglar, 1979
California	463	135(29.2)	236(51)		89(19.2)		8	Grunbaum et al., 1980
HISPANIC								
Bexar County, TX	200	(26)	(51)		(24)			Ganaway and Lux, 1978
California	161	(21.7)	(55.9)		(22.4)		9	Grunbaum et al., 1978
Miami, FL	360	75(20.8)	179(49.7)		97(26.9)	9(2.5)	4	Stuver, 1979
Los Angeles, CA	145	43(29.7)	67(46.2)		35(24.1)		4	Siglar, 1979
California	775	218(28.1)	393(50.7)		163(21)		10	Grunbaum et al., 1980
OTHER								
U.S. Japanese	23	2(8.7)	10(43.5)		11(47.8)			Harris et al., 1959
New York, NY Chinese	118	(14.8)	(38.3)		(47)	(0.03)		Parker and Bearn, 1961
Seattle, WA Oriental	494	34(6.9)	190(38.4)	0	270(54.7)	0	11	Giblett and Brooks, 1963
New York, NY Chinese	113	16(14.2)	45(39.8)		52(46)		12	Shim and Bearn, 1964a
California and Hawaii Asian	376	(7.7)	(37.8)		(53.5)		13	Grunbaum et al., 1978
California and Hawaii Asian	1,105	148(13.4)	444(40.1)		512(46.3)		14	Grunbaum et al., 1980

1. 66 Hp 1 and 2-1 were subtyped:
   4(6.1)1S-1; 5(7.6)1S-1F; 36(54.5)2-1S; 21(31.8)2-1F
2. 4 were HpCa and 2 were 'Johnson'
3. Approximately 15% of the sample were Hispanic (by surname)
4. And see Shaler (1978)
5. 222 Hp1 and 2-1 were subtyped:
   26(11.7)1S-1S; 20(9.0)1F-1F; 49(22.1)1S-1F; 61(27.5)2-1S;
   55(24.8)2-1F; 6(2.7)2M-1S; 5(2.3)2M-1F
6. 3.2% were "rare"
7. 4(0.79) were "rare"
8. 3 were "rare"
9. "Chicano/Amerindian" population
10. "Mexican" population; 1 was "rare"
11. 80 Hp1 and 2-1 were subtyped:
    14(17.5)1S-1S; 1(1.3)1S-1F; 64(80)2-1S; 1(1.3)2-1F
12. Subtyping:
    all 16 1-1 were 1S-1S; all 45 2-1 were 2-1S
13. 1.1% were "rare"
14. 1 was "rare"

SECTION 41. GROUP SPECIFIC COMPONENT

41.1 Recognition—Genetic Variation

Group specific component (Gc) was first recognized because of its polymorphism. Hirschfeld (1959b) found that normal human sera subjected to immunoelectrophoretic analysis showed qualitative differences in proteins in the α_2 globulin region. Sera could be classified into three groups, originally called "1", "2" and "3". These differences were not attributable to the Hp polymorphism, and those seen in human sera were analogous to qualitative variation seen in rabbit sera when the latter were tested by immunoelectrophoresis with a horse anti-rabbit serum (Hirschfeld, 1959a). A preliminary report on the variation in human sera was given at a meeting late in 1958 (Hirschfeld, 1960). The type "1" and "3" human sera showed single immunoprecipitin arcs, while type "2" showed both. The type "2" pattern was indistinguishable from that given by an equal mixture of type "1" and type "3" sera, and the patterns were invariant in a given person over the course of time. Family, and mother-child pair studies (Hirschfeld *et al.*, 1960) indicated that this was an inherited variation in which types 1 and 3 were homozygous, and type 2 was the heterozygote. All the observatons could be explained by a pair of alleles, and it was suggested (Hirschfeld and Beckman, 1960) that the system be called the 'group specific component', and the genetic locus controlling its expression be called *Gc*. The alleles were designated Gc^1 and Gc^2, and the system was independent of Hp, Gm, Tf and pseudocholinesterase. Types "1" and "3" thus became Gc 1-1 and Gc 2-2, respectively, and type "2" was Gc 2-1, by analogy to the nomenclature that had been developed for haptoglobin. Family and population studies soon established that the two allele hypothesis of inheritance was correct (Hirschfeld *et al.*, 1960; Nerstrøm, 1963a and 1963b; Reinskou and Mohr, 1962; Mansa *et al.*, 1963; Bütler *et al.*, 1963; Reinskou, 1965a; Seppälä *et al.*, 1967; Suyama and Uchida, 1969), and that the gene frequencies differed in racially and ethnically distinct populations (Hirschfeld and Beckman, 1961; Cleve and Bearn, 1961a and 1961b; Baitsch *et al.*, 1963; Blumberg *et al.*, 1964a; Hummel *et al.*, 1970). The earlier studies on the Gc polymorphism were well reviewed by Hirschfeld (1962).

The Gc types are detectable electrophoretically as well. In 1959, Smithies had noticed that there were differences in the migration patterns of proteins in the "post albumin" region, and thought that these might have a genetic basis (Smithies, 1959a and 1959b). In 1962, Schultze *et al.* (1962a) showed clearly that the Gc types were determinable by starch gel electrophoresis. The following year, it became clear that the Gc types and the "post albumin" variations were identical, as sera were examined both by immunoelectrophoresis and by starch gel electrophoresis (Arfors and Beckman, 1963). Gc phenotypes have been determined under a number of different conditions on starch gels (Parker *et al.*, 1963; Bearn *et al.*, 1964a) and on polyacrylamide gels (Kitchin and Bearn, 1966). Under some electrophoretic conditions, heterogeneity in the homozygous types was noticed. It was thought that this might indicate something about the subunit structure of the molecule. The matter is discussed further in a subsequent section. Azen *et al.* (1969) separated the Gc proteins on starch at high voltages, and said that better resolution was obtained, but that an additional component appeared in the 1 and 2-1 types. Gc phenotypes can be determined by a number of variations and combinations of electrophoresis and immunoelectrophoresis, as is discussed further below.

41.2 Further Gc Phenotypes

Hirschfeld (1962) found two Gc types that did not correspond to the common phenotypes. One, from the serum of an African Negro, appeared to represent Gc 1 and a faster moving component called "Y"; thus Gc 1-Y. The other, from a Caucasian serum, had a component that was faster than 2, but slower than 1, and it was called "Gc X". Parker *et al.* (1963) also reported a fast variant in a Black subject, and one of intermediate mobility in a Caucasian. Family studies could not be carried out in either of these cases to prove that the variants were reflections of other alleles at the *Gc* locus, however. The 'Caucasian' variant of Parker *et al.* (1963) is sometimes called 'Gc Caucasian' or 'Gc Cau', and it is probably the same as 'Gc X' but the two have never been directly compared (Johnson *et al.*, 1975). Cleve *et al.* (1963a) found several additional phenotypes that could be accounted for by two additional variant alleles. The first was seen in Chippewa Indians, and was called 'Gc Chippewa' or 'Gc Chip'. Gc Chip-1 was hard to distinguish by immunoelectrophoresis, but was distinctive by starch gel electrophoresis. It was due to an allele designated Gc^{Chip}. Evidence of the second allele was detected in Australian aborigine sera, which was called Gc^{Ab}. All three phenotypes, Gc Ab-1, Gc Ab-2 and Gc Ab-Ab, were observed. The Gc Ab band was faster than Gc 1 or Gc Chip, and was best detected in polyacrylamide gels (Kitchin and Bearn, 1966). No differences could be detected by Kitchin and Bearn (1966) between Gc Ab, Gc Y and another variant originally described in Greenland Eskimos by Persson and Tingsgaard (1965), either by immunoelectrophoresis or polyacrylamide gel electrophoresis, and these were thought to be the same. In a direct comparison on thin layer agarose gels, followed by immunofixation detection, however, Johnson *et al.* (1975) found that Gc Ab/Y was different

from the Eskimo variant, which they called 'Gc Esk'. In addition, 'Gc Esk' could not be distinguished from 'Gc D' (see below), and so 'Eskimo' and 'Darmstadt' may be identical. Reinskou (1965b) found another pair of variant types in Norwegian sera, which could be designated 'Norw-1' and 'Norw-2'. He did not care for the descriptive nomenclature that had developed, though, and suggested that the gene responsible for the 'Norw' phenotypes be called Gc^{1C}, and the phenotypes 1-1C ('Norw-1') and 2-1C ('Norw-2'). The 'Norw' and 'Chip' phenotypes appear to be characterized by altered proportions of the heterogeneous Gc 1-1 proteins. Gc Chip has an increased anodal portion, and Gc Norw an increased cathodal one. The "C" in the Gc^{1C} designation stands for "cathodal", and Reinskou (1965b) said that Gc^{Chip} should be designated Gc^{1A}, where "A" stands for "anodal". The Norw variants are apprently quite rare. A second example was reported by Rittner and Dahr (1969). Other variants that have been reported are: Gc^{Z}, observed as 1-Z and 2-Z (Hennig and Hoppe, 1965; Cleve *et al.*, 1966); Gc Bangkok or Gc^{Bkk}, seen as Gc 1-Bangkok (Rucknagel *et al.*, 1968); Gc Darmstadt, or Gc^{D}, seen as D-1 and D-2 (Cleve *et al.*, 1970); Gc Wien, or Gc^{W}, seen as Gc W-1 and W-2 in 8 members of a family in Vienna (Wien) (Speiser *et al.*, 1972); Gc Japanese or Gc^{J}, seen as Gc J-2 (Omoto *et al.*, 1972); Gc Opava, or Gc^{Op}, seen as Gc Op-1 and Op-2 (Cleve, 1973; Vavrusa and Cleve, 1974); Gc Boston, or Gc^{B} (if genetic transmission can be proven), seen as B-2 (Johnson *et al.*, 1975); and Gc Toulouse, or Gc^{T}, seen as Gc T-2 (Constans *et al.*, 1978).

Cleve (1973) reviewed all the known variants to date, and could classify them into four major categories: (1) Molecular variants, with electrophoretic mobility different from Gc 1 and Gc 2. The faster ones are Gc Ab, Gc D, Gc Wien and Gc Japanese; Gc Opava migrates in between Gc 1 and Gc 2; and the slower ones are Gc Bangkok and Gc Z. (2) This group included Gc Chippewa and Gc Norway variants, which, as noted above, seem to represent altered proportions of heterogeneous bands of Gc 1-1; (3) This group consisted of 'subgroups' of the Gc^{1} allele, as disclosed by a particular antiserum, raised in sheep against Gc 2-2 (Ruoslahti, 1965). Type 1 sera could be classified as 'reacting' or 'nonreacting', and the 'reactor' characteristic was inherited in the manner of a Mendelian dominant. (4) The fourth group comprised the silent alleles of *Gc*. These are apparently very rare. Henningsen (1966) reported the possibility of Gc^{0} in a family with peculiar Gc inheritance. Prokop and Uhlenbruck (1969) reported a family in which a Gc 2 father had a Gc 1 daughter, who, in turn, had a Gc 2 son. There was no question about the maternal relationship of the daughter to her son, and the easiest explanation was that the father was $Gc^{2}Gc^{0}$, his daughter $Gc^{1}Gc^{0}$, and her son $Gc^{2}Gc^{0}$. Patscheider and Dirnhofer (1979) described an extraordinary sibship in which Gc^{0} and ESD^{0} were apparently segregating together. A child in the third generation was believed to be $Gc^{1}Gc^{0}$, $ESD^{1}ESD^{0}$, and the family study indicated that the Gc^{0} had been inherited from the maternal grandfather, and the ESD^{0} from the maternal grandmother. The child's mother was also, thus, a double heterozygote for the silent alleles.

This classification of the Gc phenotypes by Cleve (1973) would have to be revised somewhat in view of the finding that there are two Gc^{1} alleles, whose products are revealed by isoelectric focusing (section 41.3). In addition, the best technique for typing most of the Gc variants seems to be thin layer agarose electrophoresis, followed by immunofixation detection (Johnson *et al.*, 1975). Their studies clarified some of the relationships between the rare variants. Patterns of some of the Gc types as seen using this technique are shown in Figure 41.1.

The majority of the Gc variant phenotypes are rare. Gc Chip reaches polymorphic frequencies in the Indians after whom it was named. Gc^{Ab}, giving rise to the Gc Ab phenotypes (likely equivalent to 'Gc Y' in Black populations), occurs in appreciable frequencies in the aborigines,

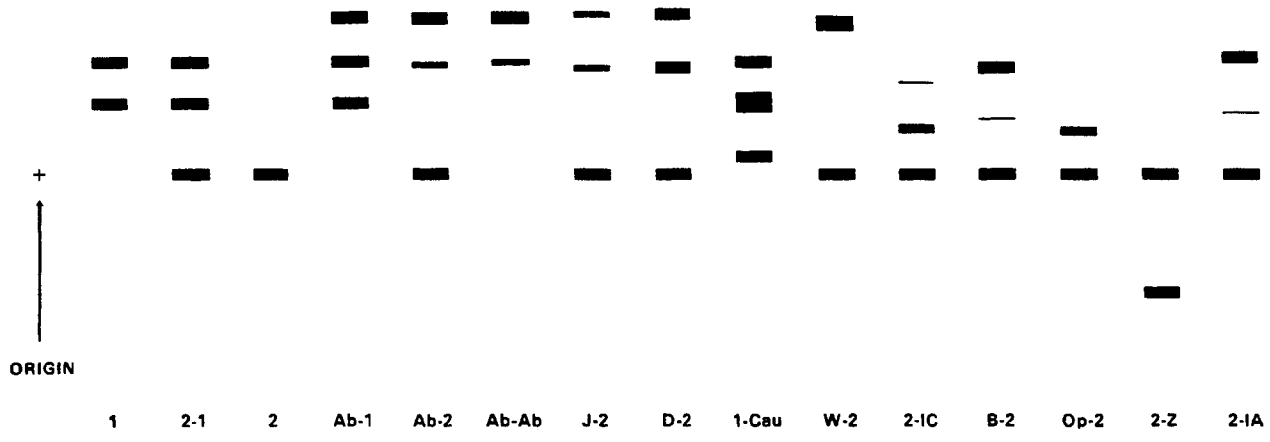


Figure 41.1 Electrophoretic Patterns of Some Gc Phenotypes (after immunofixation agarose gel electrophoresis, pH 8.6). Identities and probable identities: Gc Ab = GcY; Gc X = GcCau; Gc 2-1A = Gc 2-Norw; Gc 2-1A = Gc 2-Chip.

and is occasionally seen in Black people. There is little population data, but Gc^{Ab} had a frequency of 0.015 in 492 Black Americans from Pennsylvania and Georgia (Kueppers and Harpel, 1979). Gc Ab phenotypes are seen in various populations in Oceania as well (Kirk *et al.*, 1963).

41.3 Additional Genetic Variation at the *Gc*^{1} Locus—The Gc "Subtypes"

In 1977, Constans and Viau found that "subtypes" of Gc 1 could be distinguished by polyacrylamide gel isoelectric focusing (PAGIF), and detection of the Gc bands by immunofixation. The "fast" and "slow" bands of Gc^1 were designated "F" and "S", and accounted for by the alleles Gc^{1F} and Gc^{1S}. These Gc alleles, along with Gc^2 give rise to the phenotypes 1F, 1F-1S, 1S, 2-1F, 2-1S and 2. The Gc^{1F} frequency was comparatively low in a Pyrenean valley population, but about 0.58 in a central African pygmy group. These observations, and the proposed genetic explanation for them, have been amply confirmed (Constans *et al.*, 1978; Kühnl *et al.*, 1978; Ishimoto *et al.*, 1979; Hoste, 1979; Kueppers and Harpel, 1979). The Gc^{1F} and Gc^{1S} frequencies in Hessen were 0.125 and 0.603, respectively (Kühnl *et al.*, 1978), while in a Belgian population, they were 0.167 and 0.543 (Hoste, 1979). Constans *et al.* (1978) extended their original studies, and found two additional variants of Gc. One, found in an African pygmy population, exhibits a band in about the same position as the second band of a 1-1 on polyacrylamide gel electrophoresis. It was a variant of Gc 2, and was called '2a' (for 'anodal'). It was inherited, and the responsible allele was designated Gc^{2a}. Both 2a-1 and 2a-2 phenotyes were observed, and the former can be further classified as 2a-1F or 2a-1S by isoelectrofocusing. The other variant was found in a tribe of South American Indians from Bolivia. It was called 'Gc Am-1' and was indistinguishable from Gc Ab-1 by electrophoresis, but it was assumed that Gc Am types reflect a new allele, Gc^{Am}. Ishimoto *et al.* (1979) subtyped Gc in Japanese populations, and found that the Gc J phenotypes could be further subdivided into a series of phenotypes representing combinations of Gc^{Ja}, Gc^{Jb}, Gc^{Jc} and Gc^{Jd} with Gc^{1S}, Gc^{1F} or Gc^2. They found some further variants as well, which were called Gc Ja, Jb, Jc, Jd, Ok and Mie. In a note attached to the reprint, Dr. Ishimoto noted that these variants were equivalent to the Gc 1A2, 1A3, 1A9, 1A8, 1C2 and 1C4, respectively, of Dr. Constans. This latter nomenclature resulted from an international conference on Gc variants and nomenclature, held in Paris in 1978 (Constans and Cleve, 1979). Correspondence between old and new nomenclature for about 30 known variants was given, along with representations of the electrofocusing patterns. Briefly, the "A" and "C" designations mean "anodal" and "cathodal", and variants are then classified according to mobility. For example, Gc Ab is now Gc 1A1, and the phenotype that would previously have been called Gc 'Ab-1F' is now Gc '1A1-1F'. The paper must be read by those interested in Gc typing. Equivalences are shown in Table 41.1.

41.4 Methods of Phenotyping Gc

A number of procedures have been employed to distinguish the Gc phenotypes in serum. The three common phenotypes, 1, 2-1 and 2, can be diagnosed with any of them in experienced hands. Some of the rarer variant types are more difficult to discriminate by certain methods, and easier using other ones, and this has been one of the difficulties in the unequivocal classification of the variants observed in different laboratories using different methods. The original procedure, immunoelectrophoresis, serves well in many laboratories (see in Hirschfeld, 1968a). Interpretation of the patterns is made considerably easier if specific anti-Gc serum is used rather than anti-human serum. Both starch and polyacrylamide gel electrophoresis have been used for typing Gc, as discussed above. A polyacrylamide disc gel procedure was devised by Raunio *et al.* (1966). Polyacrylamide gel electrophoresis can be employed on a preparative

Table 41.1 Gc Variant Nomenclature Equivalents

New Gc Nomenclature ★	Old Designations
1S	1S
1F	1F
2	2
1A1	Ab;Y;☆
1A2	Or;◇ J; OMS 25;◇ Ja●
1A3	OMS 6;◇ OMS 22;◇ N; Jb●
1A4	Igloo; Y;●◇ Eskimo
1A5	V²◇
1A6	1C◇
1A7	Op,
1A8	Am1;◇ TK2;◇ OMS 10;◇ Jd●
1A9	Am2; TK1;◇ OMS 14;◇ Jc●
1C1	T;
1C2	OMS 1;◇ Ok●
1C3	1B; V¹◇
1C4	OMS 21;◇ Mie●
1C5	Bagnère◇
1C6	1D1◇
1C7	1D2;◇ Op,
1C8	1E
1C9	SI
1C10	1G◇
2A1	2C◇
2A2	Op,
2A3	2A
2A4	2Y
2A5	V²;◇ 2B
2A6	Wien
2C1	2D◇
2C2	Z

★ According to Constans and Cleve (1979); only one gene product is shown; only variants actually compared by isoelectric focusing are shown

☆ The "Y" seen in Black populations is thought to be equivalent to "Ab"

● Seen in Japanese ● According to Ishimoto et al., (1979)

◇ Familial transmission not clearly established

scale (Simons and Bearn, 1967). Prokop (1963) described a kind of two-dimensional immunoelectrophoretic procedure suitable for Gc. Electrophoresis was performed as usual, but then two antibody troughs were cut in parallel on either side of the sample position, and electrophoresis performed at 90° to the original direction. This procedure was much faster than classical immunoelectrophoresis, in which the antibodies are allowed to diffuse into the gel. The antigen-antibody crossed electrophoresis procedure of Laurell (1965), which is described in detail by Weeke (1973), has also been used for Gc typing (see in section 2.4.3.2). Sutcliffe and Brock (1973) used this technique, and it has also been found useful in comparing Gc variants. In 1969, Alper and Johnson described a technique called immunofixation electrophoresis (section 2.4.2); the technique is further discussed by Ritchie and Smith (1976). Here, the separated components are allowed to react with specific antibody in the gel, after which the gel is washed free of excess protein, and the specific complexes stained. This technique has been used extensively for the detection of Gc types following electrophoresis (Johnson *et al.*, 1975; Martin and Kopietz, 1976; Grunbaum and Zajac, 1977; Wraxall and Stolorow, 1978). Immunofixation may also be used to detect Gc following isoelectric focusing. Most of the techniques that have been found suitable for bloodstain typing utilize some form of immunofixation detection scheme (see below). Hoste (1979) detected the Gc bands on polyacrylamide gels after isoelectrofocusing by a simple non-immune sulfosalicylic acid staining procedure.

41.5 Physiological and Biochemical Studies on Gc

41.5.1 The function and properties of Gc protein

For years, Gc proteins were a genetic curiosity, the system being well studied as a genetic marker, but the protein constituents of it having no known physiological function. It has been known for many years that serum contains some sort of Vitamin D binding protein, and that this protein is an α-globulin (Thomas *et al.*, 1959). Belsey *et al.* (1974) found that rat and chick, in addition to human, sera all have this protein, and that it binds 25-hydroxy-Vitamin D preferentially. The Vitamin D binding protein was isolated from human serum and partially purified by Imawari *et al.* (1976). Daiger *et al.* (1974 and 1975a) then observed polymorphism in the human serum Vitamin D binding protein, and quickly realized that they had 'rediscovered' the Gc polymorphism. Their studies indicated that Gc and Vitamin D binding proteins are identical. Bouillon *et al.* (1976) rapidly confirmed the identity using an extensively purified preparation. They said that the protein should be renamed "transcalciferin". Haddad and Walgate (1976) purified the serum Vitamin D binding protein, and likewise showed that it was immunologically identical to Gc. Cleve and Patutschnick (1977) used autoradiography to show that the components of the common Gc phenotypes, as well as those of many of the variants, bind Vitamin D_3. No differences in Vitamin D_3 binding were detected among the variant types. The MW of the purer preparations of Gc is in the neighborhood of 52,000 (Imawari *et al.*, 1976) to 58,000 (Bouillon *et al.*, 1976).

There have been various reports of the Gc level in normal serum. Published values range from about 0.28 to about 0.75 mg per mℓ of serum. The higher values are those of Kitchin and Bearn (1965) using quantitative immunoprecipitation, and the lowest values were obtained by radial immunodiffusion measurements (Kueppers and Harpel, 1979), who used a commercial standard serum for calibration. There have been suggestions, too, that the normal Gc content of serum differs according to phenotype, but this variation was not seen by Kitchin and Bearn (1965) nor by Kueppers and Harpel (1979).

41.5.2 Biochemical studies

The amino acid composition of Gc has been studied by Cleve *et al.* (1963b), Bowman and Bearn (1965) and Bowman (1967 and 1969) among others. The fast and slow bands of 1-1 and 2-2 are all very similar, and the Asp and Glu content is quite high. The molecule contains carbohydrate but little is known about how it is arranged. Schultze *et al.* (1962b) found the equivalent of 7 hexose, 5 acetylhexosamine and 1 fucose per 51,000 MW, but no sialic acid. The data of van Baelen *et al.* (1978), however, strongly suggest that the Gc 1 molecule must contain sialic acid.

Bowman (1969) proposed a molecular model for Gc, based on end group analysis, peptide maps and other chemical data. The model is further discussed in the review by Putnam (1977) as well. The molecule is a dimer, according to her proposal, which can be made up of three different kinds of chains controlled by three separate genetic loci. The chain called δ is common to fast bands of both 1-1 and 2-2. In 1-1, δ is combined with α^1 to give $\alpha^1\delta$ which is characteristic of 1-1. In 2-2, δ is paired with an α^2 chain to give the $\alpha^2\delta$ characteristic of 2-2. A β chain combines with α^1 to give the $\alpha^1\beta$ slow band of 1-1. Van Baelen *et al.* (1978) carried out studies on the Gc proteins as separated by isoelectrofocusing. Gc 2-2 contained a single protein, where Gc 1-1 had two bands with lower pI. The 2-1 contained all three. An anodal shift was observed after incubation of the apo-Gc with excess 25-hydroxy-Vitamin D. Sialidase treatment had no effect on Gc 2-2, but the faster Gc 1-1 band focuses in the position of the slower band after treatment, strongly indicating N-acetyl neuraminic acid in the Gc 1-1 fast band.

Reviews of the Gc polymorphism may be found in Cleve and Bearn (1962), Bearn *et al.* (1964b), Reinskou (1968a), Giblett (1969), Prokop and Uhlenbruck (1969) and Putnam (1977).

41.6 Medicolegal Applications

41.6.1 Disputed parentage

The Gc polymorphism has been used in disputed paternity cases in a number of laboratories for quite some time (Hirschfeld and Heiken, 1963; Nerstrøm, 1963a; Ritter,

1963; Marek *et al.*, 1963; Bütler *et al.*, 1963; Holzhausen *et al.*, 1964; Reinskou, 1966b). The probability of excluding a falsely accused father of Western European origin is about 15–16% and the system is considered reliable (Reinskou, 1966a). In U.S. populations, the exclusion probabilities are about 16% for falsely accused White fathers, and about 7–8% for falsely accused Black fathers (Chakraborty *et al.*, 1974; Dykes and Polesky, 1978). The system is apparently employed only rarely in disputed parentage cases in this country (Polesky and Krause, 1977). The exclusion probabilities given above are based on the detection of the three common phenotypes. Serum phenotyping is generally straightforward. The possibility of rare or silent alleles, though they are not often encountered, must be kept in mind. If isoelectrofocusing equipment is available and the *Gc*^{1} subtypes can be determined, the exclusion probability is increased significantly. Kühnl *et al.* (1978) indicated that the figure would be 24% in the Frankfurt population; Hoste (1979) said it would be almost 32% in Belgium.

41.6.2 Gc phenotyping in bloodstains

The earlier investigators used immunoelectrophoresis for Gc typing, since this technique was the only one available for several years. A number of efforts were made to type Gc in bloodstains by this procedure, but most of them were not very successful. More recent applications of electrophoresis and immunofixation detection techniques have been more productive.

In 1963, Nerstrøm and Skafte Jensen reported that only the very freshest bloodstains could be typed for Gc using immunoelectrophoretic procedures. Peculiarly, serum stains could be grouped for several weeks. Vogt (1963) in Prof. Dr. Prokop's institute had similar results, although he could type stains up to about 10 days old made on glass plates. Heifer and Bolkenius (1966) found, however, that Gc seemed to be a little more stable in stains made on glass than in those on other substrata. There seem always to have been some difficulties with older bloods, or sera that have been transported over great distances (as is sometimes done in population studies), when immunoelectrophoresis is used for the Gc typing. Problems associated with stored, older sera were discovered by Nerstrøm (1963b) and Persson and Tingsgård (1966). There is a distinctive decrease in the quality of the precipitin arcs in these samples. Nerstrøm (1963c and 1964) was intrigued by the fact that the Gc types in serum stains were more stable than in whole bloodstains, and that old, hemolyzed blood gave typing problems that separated serum did not. The lysed cells were thought to have an adverse effect on the Gc proteins, and studies showed that lysed leucocyte and thrombocyte material did disturb the Gc pattern. A similar effect could be induced with proteolytic enzymes and products from yeast. Heifer and Bolkenius (1966) were able to type most of a series of bloods kept stored for two years, and about half the post mortem samples they attempted. They too found that serum stains could be grouped a little longer than whole blood stains on linen. Similar observations were made by Brzecka and Mikulewicz (1966). They said that a major difficulty was in concentrating the protein in bloodstain extracts to a sufficient extent that the Gc types were detectable. Gc could be typed in fairly fresh post mortem blood, but older hemolyzed samples were unsatisfactory. Tumanov and Il'ina (1974) utilized an immunoelectrophoretic technique, but with a monospecific anti-Gc serum. Stains on a variety of substrata could be typed for up to 18 months time, a considerable improvement compared with the older procedures.

In 1975, Wraxall reported briefly on efforts to apply the antigen antibody crossover electrophoresis technique to bloodstains. Apparently, many of the problems encountered in immunoelectrophoretic techniques are alleviated by the use of electrophoretic separations followed by immunofixation detection. Johnson *et al.* (1975) said that they had no difficulty typing aged, contaminated or jaundiced sera in this way, in contrast to the difficulties reported previously when immunoelectrophoretic techniques were being used. They preferred agarose gels for immunofixation electrophoresis over polyacrylamide or starch gel media. Martin and Kopietz (1976) reported that immunofixation electrophoresis in agarose had helped to clarify the phenotypes of some samples which were originally typed by immunoelectrophoresis. One serum typed as a 2–1 in agarose, but had looked like a "1-variant" of some kind by immunoelectrophoresis. Similarly, a specimen thought to be a possible 2-W by its immunoelectrophoretic behavior turned out to type as a Gc 2-Ab in agarose. Immunofixation may be used in conjunction with cellulose acetate membrane electrophoresis, as well as with electrophoresis in gel media. Grunbaum and Zajac (1977) described such a procedure, which needed only 20 minutes of electrohoresis to achieve separation. This technique could be used to type bloodstains up to 2 months old (Zajac and Grunbaum, 1978). A chloroform extraction technique was used to remove the hemoglobin degradation products from the bloodstain extract prior to electrophoresis. Wraxall and Stolorow (1978) described an immunofixation agarose electrophoresis typing procedure, which is fully applicable to bloodstain typing as well. The procedure is currently used in several laboratories that we know of, and yields satisfactory results on stains up to a number of months old.

Gc is not detectable in semen by radial immunodiffusion techniques which would detect it in 2.5% solutions of serum (Blake, 1976; Blake and Sensabaugh, 1976 and 1978a). If the serum level of Gc is taken to be 800 $\mu g/m\ell$, therefore, Gc would have to be present in concentrations of less than about 20 $\mu g/m\ell$ to go undetected. The serum concentration of Gc may actually be lower than 800 $\mu g/m\ell$ (section 41.5.1). The Gc system is not, therefore, a useful genetic marker in semen. Gc cannot be detected in healthy urine by immunoelectrophoretic analysis, but it can sometimes be determined in pathological (proteinuric) specimens (Nielsen *et al.*, 1963). Thus, while it is highly unlikely that Gc could be detected in urine stains, it is equally unlikely that urine contamination would interfere with Gc bloodstain typing.

41.7 Distribution of Gc Phenotypes in U.S. Populations

The data is shown in Table 41.2. The worldwide distribution of the common Gc^1 and Gc^2 alleles was discussed by Cleve (1973) and Walter and Steegmüller (1969). Gc^2 is about 0.25 to 0.30 in most European populations, and quite a bit lower in most Black African populations (of the order of 0.1). In most Asians, the frequencies are closer to those seen in Europeans. Gc^1 subtyping is comparatively recent, and there is not very much data as yet. It appears, however, that 75–85% of European Gc^1 genes are Gc^{1S}. In Black populations, Gc^{1S} is a much lower fraction of Gc^1 alleles, perhaps 15–25%. In Japanese populations, the fraction is of the order of 35%.

Table 41.2 Distribution of Gc Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) 1	2-1	2	Note	Reference
CAUCASIAN						
New York, NY	122	63(51.6)	49(40.2)	10(8.2)	1	Cleve and Bearn, 1961a
New York, NY	86	48(55.8)	32(37.2)	6(6.97)		Cleve and Bearn, 1961b
Southeastern GA	292	147(50.3)	114(39.0)	31(10.6)		Blumberg et al., 1964
Boston, MA	407	234(57.5)	146(35.9)	27(6.6)		Murray and Robinson, 1968
Tecumseh, MI	7,658	3,910(51.06)	3,139(40.99)	609(7.95)		Schreffler et al., 1971
California	4,488	(50.2)	(41.0)	(8.5)	2	Grunbaum et al., 1978
Detroit, MI	503	242(48.11)	213(42.35)	45(8.95)	3,16	Stolorow et al., 1979
Miami, FL	365	168(46)	160(44)	37(10)	16	Stuver, 1979
Los Angeles, CA	109	59(54.1)	47(43.1)	3(2.8)	16	Siglar, 1979
Southeastern PA	110	59(53.6)	40(36.4)	11(10)	4	Kueppers and Harpel, 1979
California	1,050	537(51.1)	429(40.9)	74(7)	5	Grunbaum et al., 1980
NEGRO						
New York, NY	144	115(79.86)	28(19.4)	1(0.69)	1	Cleve and Bearn, 1961a
New York, NY	120	98(81.67)	19(15.83)	3(2.50)		Cleve and Bearn, 1961b
Southeastern GA	231	192(83.1)	38(16.5)	1(0.4)		Blumberg et al., 1964
California	832	(74.5)	(21)	(2)	6	Grunbaum et al., 1978
Detroit, MI	504	366(72.62)	112(22.22)	4(0.79)	7,16	Stolorow et al., 1979
Miami, FL	339	263(77.6)	64(18.9)	6(1.8)	8,16	Stuver, 1979
Los Angeles, CA	43	34(79.1)	7(16.3)	2(4.7)	16	Siglar, 1979
Southeastern GA	219	185(84.5)	26(11.8)	4(1.8)		
Philadelphia, PA	273	198(72.5)	61(22.3)	3(1.1)	9	Kueppers and Harpel, 1979
Combined	492	383(77.8)	87(17.7)	7(1.42)		
California	867	638(73.6)	183(21.1)	18(2.1)	10	Grunbaum et al., 1980
HISPANIC						
California	1,417	(59.1)	(35.3)	(5.2)	11	Grunbaum et al., 1978
Miami, FL	360	207(57.5)	126(35)	24(6.7)	12,16	Stuver, 1979
Los Angeles, CA	102	83(81.4)	17(16.7)	2 (1.9)	16	Siglar, 1979
California	1,908	1,160(60.8)	655(34.3)	80(4.2)	13	Grunbaum et al., 1980
OTHER						
New York, NY (Chinese)	117	69(59)	42(35.9)	6(5.1)	1	Cleve and Bearn, 1961a
California and Hawaii (Asians)	3,043	(50.4)	(39.2)	(6.4)	14	Grunbaum et al., 1978
California and Hawaii (Chinese and Japanese)	1,566	780(49.8)	590(37.7)	118(7.5)	15	Grunbaum et al., 1980

1. Calculated from gene frequencies
2. 0.4% were "rare"
3. One was Gc 1-Y
4. Subtyping: 3(2.7) 1F, 19(17.3) 1F-1S, 37(33.6)1S, 8(7.3)2-1F and 32(29)2-1S
5. 10 were "rare"
6. 2.4% were "rare"
7. 18(3.57) were Gc 1-Y, 3(0.6) were Gc 2-Y and 1 was "rare"
8. 6(1.8) were Gc 1-Y
9. Subtyping:
   GA — 141(64.4) 1F, 40(18.3) 1F-1S, 4(1.8) 1S, 21(9.6) 2-1F, and 5(2.3) 2-1S;
   PA — 130(47.6) 1F, 58(21.2) 1F-1S, 10(3.7) 2S, 51(18.7) 2-1F, and 10(3.7) 2-1S;
   Combined — 271(55.1) 1F, 98(19.9) 1F-1S, 14(2.8) 1S, 72(14.6) 2-1F and 15(3.0) 2-1S;
   in PA, there were 6(2.2) Ab-1F, 3(1.1) Ab-1F and 2(0.7) Ab-2;
   in GA, there were 3(1.4) Ab-1F and 1(0.5) Ab-2.
10. 28 were "rare"
11. Population was "Chicano/Amerindian; 0.5% were "rare"
12. 2(0.6) were Gc 1-Y and 1(0.3) was Gc 2-Y
13. Population was "Mexican" and many samples were from Mexico City; 13 were "rare"
14. 4% were "rare"
15. 78 were "rare"
16. And see Shaler, 1978

SECTION 42. TRANSFERRIN (SIDEROPHILIN)

42.1 Introduction

Most of the acid-soluble iron in blood plasma is reversibly associated with a special iron transporting protein, which shuttles Fe^{3+} to marrow, and to organs such as the liver. The presence of such a protein was suspected for decades. Starkstein and Harvalik (1933) localized it as a globulin protein, and Vahlquist (1941) found that it had the electrophoretic mobility of a β-globulin. Holmberg and Laurell (1945) and Schade and Caroline (1946) independently established that a specific protein in plasma was responsible for the iron-binding properties. They also established the physiological role of the protein, and recognized its clinical importance. Schade *et al.* (1949) called the protein "siderophilin", and showed that it took up two Fe^{3+} per mole, the uptake of one anion (HCO_3^-) for each iron being necessary for the binding to take place. Ehrenberg and Laurell (1955) carried out magnetic measurements on Fe-transferrin which indicated that the iron was ferric, and ionically bound. Holmberg and Laurell (1947) established that the protein bound copper ions as well, but that the binding was much weaker than with ferric ions which would readily displace copper. They suggested that the iron binding protein be called "transferrin".

42.2 Genetic Variation

In 1956, Smithies and Poulik introduced two-dimensional electrophoresis, the initial run being carried out on a filter paper strip, which was then inserted into a starch gel for electrophoresis in the second direction, at right angles to the first. Further studies (Poulik and Smithies, 1958) indicated several components in the β-globulin region, the most heavily staining one being third in its migration rate. This band was designated "C" for that reason. In 1957, Smithies reported that a β-globulin had been found in the sera of two Black women from New York (out of 49 tested) and in the sera of 5 Australian aborigines (out of 23) which had not previously been seen. Two-dimensional electrophoresis was necessary to see these differences. In most sera, β-globulins called A, B, C, and E were observed, but in the sera just mentioned, a "D" component was found as well. This D had not been observed in several hundred sera from Caucasians. Horsfall and Smithies (1958) extended these studies to a larger sample of aboriginal sera, including samples from members of families. The original sera had exhibited approximately equal amounts of components C and D, but a few were seen in the later sample that had only D. It was suggested that these β-globulins were under the simple Mendelian genetic control of a pair of alleles, called β^C and β^D. The "C" and "D" type sera represented the homozygotes, and the "CD" sera were from heterozygotes. In five out of 420 Canadian Caucasian sera, Smithies (1958) found a slightly faster protein called "B". Family studies indicated that B was conditioned by a third allele, β^B. It was soon established that the β-globulin protein exhibiting this genetically controlled variation was equivalent to transferrin (Smithies and Hiller, 1959). Independent lines of evidence based on independent experiments by Pert, Poulik, Allison, and by Sutton and Bishop (communicated to Smithies and Hiller, 1959) all pointed to the β-globulin-transferrin identity. The locus was, therefore, named *Tf*, and the known alleles Tf^C, Tf^D and Tf^B. Harris *et al.* (1958b) found two more variants, in addition to those seen by Smithies and coworkers. The faster one was called "D_2" (Smithies' "D" becoming "D_1"), and the slower one was called "B_2" (Smithies' "B" becoming "B_1"). The subscripts were designed to classify the faster and slower (than C) bands according to electrophoretic mobility. Giblett *et al.* (1959) then found three new transferrins (as heterozygotes of C), CD_0, CD_3 and B_0C. In 1960, Harris *et al.* found a CD_4 in two European families. By this time, it had become apparent that the nomenclature scheme which had been started was not going to be able to absorb the flow of new variants. Parker and Beam (1961b) found a Tf in Navajo Indians that migrated between B_0 and B_1. This one was named "B_{0-1}", and was observed as a CB_{0-1}. Two further variants were found in New York Oriental populations by Parker and Beam (1961a). One was a B_3D in a Japanese. The other, in a Chinese, ran very much like D_1, but rather than try to continue the numbering system, it was called "D_{Chi}". Some of the variants found subsequently were still given numerical subscripts, while others took on the names of the places or populations in which they were seen. The basic symbols "C" for the usual Tf, "D" for the slower, and "B" for the faster variants, were kept. Giblett (1969) suggested dropping numerical subscripts and sticking to geographical designations. Ultimately, as the chemical structures of the variants are worked out, a nomenclature descriptive of the chemical change can be adopted (Putnam, 1975d), as was done for the hemoglobins (section 38).

References to some further variants are: B_{1-2}, seen in a Venezuelan family as CB_{1-2} (Arends *et al.*, 1962); B_{Lae}, seen as CB_{Lae} and homozygous in New Guinea (Lai, 1963); $D_{Adelaide}$, seen in Australia and perhaps identical to D_0 (Cooper *et al.*, 1964); $B_{Atalanti}$, seen in Greece, and apparently not clearly distinguished from B_1 (Murray *et al.*, 1964); D_{Wigan}, seen in England (Glen-Bott *et al.*, 1964); $D_{Finland}$, seen in Finns, and perhaps the same as D_2 (Seppälä, 1965; Seppälä *et al.*, 1967); $B_{Lambert}$, perhaps the same as B_{1-2} (Barnett and Bowman, 1968); D_{Madiga}, $D_{Mudiraj}$, $B_{Goldsmith}$ and B_{Madiga}, seen in India (Rao *et al.*,

1979). There are several other variants as well, and further characterization will be required in many cases to demonstrate which of them are actually identical. Some of them are very rare, and serum is not available for the comparisons. Variants are discussed by Kirk (1968b), Bowman (1968), Giblett (1969) and Putnam (1975d). Relative electrophoretic mobilities of the Tf variants are indicated diagrammatically in Figure 42.1.

The variants can be arranged on a scale of relative electrophoretic mobility, as has been done by Sutton and Jamieson (1972). They classified many of the Tf variants according to their relative mobilities on polyacrylamide disc gel electrophoresis, setting the mobility of C = 0. This kind of scheme provides a basis for comparing newly discovered variants with older ones, even if one does not have access to panels of sera containing variants, assuming the exact same electrophoretic procedures are followed. A representation of this scheme is shown in Figure 42.2. In 1975, Rittner and Rittner suggested a variation of this approach. They compared a number of variants by high voltage electrophoresis on agarose gels, and in longer runs could resolve most of them. They wanted to use Tf B_2 as the "reference variant", setting its relative electrophoretic mobility equal to +0.7 for practical reasons. The variants could then be designated according to their mobilities, e.g. B 0.78, D 1.06, etc. This nomenclature is analogous to that adopted for C3 variants (section 45). This nomenclature scheme is also shown in Figure 42.2.

The many population and family studies which have been carried out are fully consistent with the variant forms being representatives of alleles at the *Tf* locus (e.g. Kirk *et al.*, 1964; Kurz and Ritter, 1972). At least two families have been studied in which two non-C Tf genes occurred in heterozygous combination in some members. Beckman (1962) found a B_2D_1 combination, the first in which "fast" and "slow" variant types occurred together. Robinson *et al.* (1963) described an individual of $B_{1-2}B_2$ phenotype.

There are several reports of families with Tf deficiency (Heilmeyer *et al.*, 1961; Goya *et al.*, 1972). Other reports have been discussed by Gitlin and Gitlin (1975). These families suggest the possibility of a rare silent allele at *Tf*, although the data are somewhat complicated, and other genetic explanations are possible.

42.3 Further Genetic Heterogeneity in Tf—Tf C Subtypes

In 1978, Kühnl and Spielmann (1978a) in Germany, and, somewhat independently, Thymann in Denmark demonstrated that the common transferrin, Tf C, was resolved into three patterns by isoelectric focusing on polyacrylamide gels (PAGIF). Family studies revealed that these patterns represented three phenotypes, called Tf C1, C2-1 and C2, due to a pair of codominant alleles Tf^{C1} and Tf^{C2}. Thymann (1978) found the same polymorphism in Denmark. Apparently, Kühnl and Spielmann had first reported at a conference (the 7th International Congress of Forensic Haemogenetics in Hamburg) that this was some kind of an Hp polymorphism. She called the alleles Tf^1 and Tf^2. Using Kühnl and Spielmann's nomenclature, the German sample showed $Tf^{C1} = 0.8195$ and $Tf^{C2} = 0.1720$, while the Danish one showed $Tf^{C1} = 0.81$ and $Tf^{C2} = 0.19$. The Tf^{C1} of Kühnl and Spielmann is the Tf^2 of Thymann. Kühnl and Spielmann (1978a) showed that this was not a sialic acid effect, for neuraminidase did not obliterate the three phenotype behavior of the samples. The new Tf C subtypes were quickly confirmed by Stibler *et al.* (1979), who had earlier noticed heterogeneity of Tf C upon isoelectric focusing (Stibler *et al.*, 1978), and by Hoste (1979). In 1979, Kühnl and Spielmann (1979a) found a third allele, which was called Tf^{C3}. Phenotypes conditioned by the third allele were detected by a modified PAGIF procedure using a shallower pH gradient. The gene frequencies in the extended system were $Tf^{C1} = 0.795$, $Tf^{C2} = 0.155$ and $Tf^{C3} = 0.042$. There are, thus, six subphenotypes: Tf C1, C2, C3, C2-1, C3-1 and C3-2. Family studies indicated the accuracy of this genetic model. In 1979, Kühnl *et al.* looked at a series of Tf B and Tf D heterozygotes on PAGIF. In all CB or CD samples, only C1 or C2 was seen, but never both, suggesting that the new series of Tf^C alleles are true alleles of those conditioning the B and D variants. If all these *Tf* genes are indeed allelic, then any of the variant alleles discussed in section 42.2 could be paired with Tf^{C1}, Tf^{C2} or Tf^{C3}, increasing the number of possible Tf phenotypes by three-fold. Transferrin genetic variant nomenclature is due for some standardization in view of the new findings.

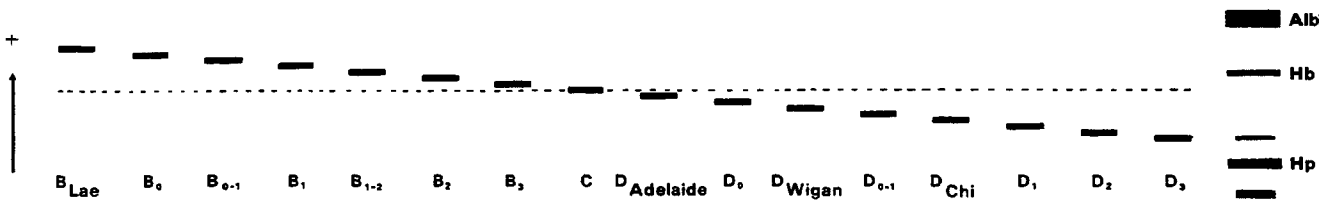


Figure 42.1 Relative Electrophoretic Mobility of Some Tf Variants at Alkaline pH.
Dotted line indicates position of the Common Tf C.

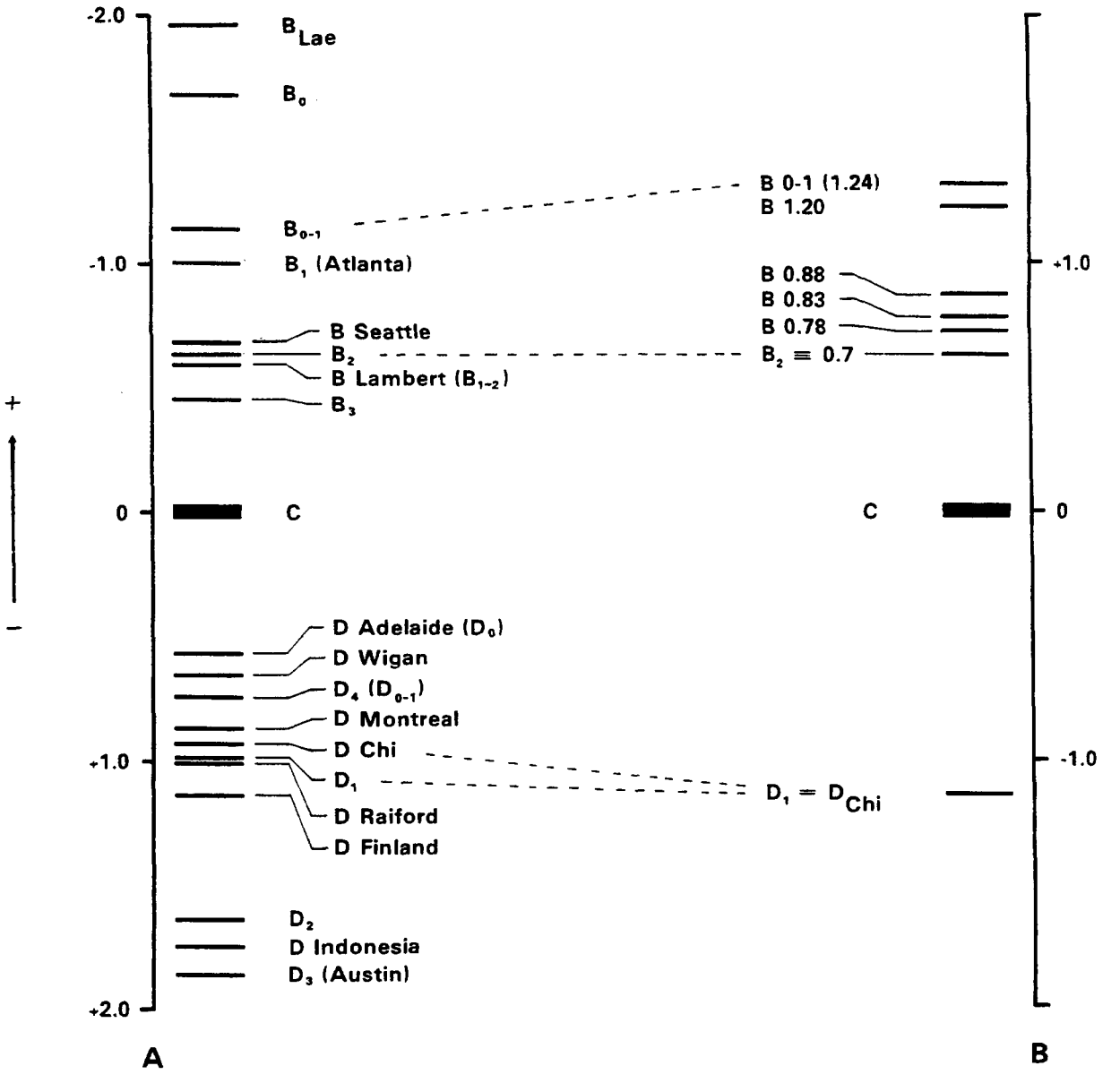


Figure 42.2 Arrangements of Transferrin Variants according to Relative Electrophoretic Mobility on (A) Polyacrylamide Gels (Sutton and Jamieson, 1972) and (B) Agarose Gels after High Voltage Runs (Ritner and Ritner, 1975).

42.4 Biochemical Studies on Transferrin

42.4.1 Structure of transferrin

There have been many studies on the amino acid composition of human Tf, which are reviewed by Putnam (1975d). There is strong evidence that the Tf molecule is a single polypeptide chain (Greene and Feeney, 1968; Mann *et al.*, 1970; MacGillivray and Brew, 1975) of MW about 76,000–77,000 (about 630 amino acid residues). There are 15 or 16 intrachain disulfide bridges, and apparently no free -SH groups. The molecule can be cleaved with cyanogen bromide to yield 9 fragments (see in MacGillivray and Brew, 1975, and Putnam, 1975d), and a number of these have been sequenced. Brune *et al.* (1978) reported the sequence of the N-terminal peptide fragment (50 residues), which they said completed about a third of the total sequence. The N-terminal peptide fragment contained two intrachain disulfide bridges, between Cys9 and Cys48, and between Cys19 and Cys39. There is considerable similarity in the composition of human and animal transferrins (Palmour and Sutton, 1971; Graham and Williams, 1975). There is also quite a bit of structural homology in different segments of human Tf C (MacGillivray and Brew, 1975), and between Tf and lactoferrin (Metz-Boutique *et al.*, 1978).

Transferrin is a glycoprotein, and its carbohydrate structure has been worked out completely (Jamieson *et al.*, 1971). There are two identical carbohydrate chains per Tf molecule, attached through an internal GlcNAc residue to Asn in the peptide chain. Each carbohydrate chain has two N-acetyl neuraminic acid "ends", so that there are four in the complete molecule. The structure is shown in Putnam (1975d) as well as in the original paper. Graham and Williams (1975) compared the carbohydrate structure of human Tf with that of the transferrin from several animal species. The presence of terminal sialic acid residues in human Tf was recognized early (Parker and Bearn, 1961c and 1962). Action of sialidase alters the mobility of Tf C in stepwise fashion, yielding five different bands which appear to differ from one another by a single unit of negative charge (Parker and Bearn, 1961c). This result shows that all four sialic acid residues are accessible to the sialidase. Sialidase treatment of Tf variants yielded the same kind of behavior observed with Tf C (Parker and Bearn, 1962). Similarly, neuraminidase treatment alters the pI of the Tf C subtypes (Kühnl and Spielmann, 1978a). However, neither the sialic acid nor the complete carbohydrate moiety appears to have anything directly to do with the polymorphism, which almost certainly reflects peptide chain compositional differences. There is evidence that the carbohydrate moiety has a biological role in Tf function (see in Putnam, 1975d).

42.4.2 Structural differences among Tf variants

Much work remains to be done in this field, and it is made more difficult by the size and internal complexity of the Tf molecule in terms of amino acid composition and by the rarity of many variants. Most of the information is based on differences in peptide fingerprints of a few of the variants. Wang and Sutton (1965) indicated that D_1 differed from C by an Asp→Gly substitution, which was detected in chymotryptic digests. This same substitution is found in Tf D_1 samples from American Blacks or from Australian aborigines (Wang *et al.*, 1967). Wang *et al.* (1966) found that on the basis of tryptic digest patterns, Tf B_2 differed from Tf C by a single Gly→Glu substitution. A simple substitution of His→Arg was reported for D_{Chi} by Wang *et al.* (1967). It now appears (Sutton *et al.*, 1975) that Tf D_1 may have two substitutions, Asp→Gly and Asn→Gly, while D_{Chi} has only the one. Immunological differences between Tf C, D_1, D_{Chi} and D_3 were not detected by Wang *et al.* (1968) using a radioimmune inhibition of precipitation technique.

Most authorities are inclined to think that the variants derive from one-step mutations resulting in single amino acid changes. There are some serious difficulties with such a view (Putnam, 1975d). It is hard to explain the large number of Tf variants, which appear to differ by at least a single unit of charge, by single amino acid substitutions in a molecule that appears to have no subunit structure. It is possible that there are size differences, which are reflected in the electrophoretic mobility, but there is no evidence for significant size differences. Perhaps more extensive structural differences do exist, although if these are different single amino acid substitutions, double or multiple mutations might have to be invoked as an explanation. Deletions or additions are possible; another possibility is that of repeating sequences within Tf.

42.4.3 Metal binding properties

This subject is complex, and reviewed in more detail by Putnam (1975d). Each Tf molecule binds two iron atoms (Schade *et al.*, 1949), and these are Fe^{3+} (Ehrenberg and Laurell, 1955). Bonding is ionic and bicarbonate anion is taken up for each ferric ion bound. Further, the binding is pH dependent. Tf can combine loosely with other metals, such as Cu (Holmberg and Laurell, 1947; Aasa *et al.*, 1963). Earlier work tended to suggest that the binding sites in Tf were independent and identical (e.g. Aisen *et al.*, 1966). More recent evidence indicates, however, that they are not identical (Harris, 1977; Aisen *et al.*, 1978). The sites are designated "A" and "B" (or "a" and "b"). Makey and Seal (1976) showed that four transferrins, representing iron free Tf (apo-Tf), Tf with Fe bound to site A, Tf with Fe bound to site B, and Fe_2-Tf, could be separated by electrophoresis in polyacrylamide gels containing 6M urea in Tris-EDTA-borate buffers at pH 8.4. It was not clear just exactly why the forms could be separated in this way. These results may help to explain the immunological differences seen by Jager and Gubler (1952) and Kourilsky and Burtin (1968). Leibman and Aisen (1979) took advantage of the ability to separate the forms in order to estimate their relative amounts in normal sera. Binding was not random, nor was it determined by the relative binding strengths of the A and B sites. The more acid-labile and weakly-binding B site of Tf was predominantly occupied.

42.5 Medicolegal Applications

The Tf system is used in some laboratories in disputed parentage cases (Mauff *et al.*, 1975; Dykes and Polesky, 1978). The exclusion probability is low in Europeans and in American Caucasians (about 1%), and about 5% in Black Americans. Chakraborty *et al.* (1974) quoted slightly higher values. The finding that Tf C can be subtyped by electrofocusing techniques, however, and that the alleles are reasonably well distributed (at least in Europeans), greatly enhances the value of the system. The exclusion probability increases to about 13–15% in Europeans if Tf C is subtyped (Thymann, 1978; Kühnl and Spielmann, 1979a; Hoste, 1979). Use of the Tf C subtypes is made more economical by the fact that the *Gc*^{1} subtypes (section 41.3) and the *Tf*^{C} subtypes can be determined in the same isoelectric focusing gel (Thymann, 1978; Hoste, 1979).

Turowska (1969) noted that Tf could be typed in fresh bloodstains. There are a few laboratories in which Tf typing is done in bloodstains at the present time. It is possible to type Tf in some stains which are a matter of months old. The commoner variants can be detected on agarose gels, followed by immunofixation detection, and Tf typing is normally combined with Gc typing in the same gel.

Tf can be detected in a variety of human tissues (Mason and Taylor, 1978). Transferrin can also be detected in seminal fluid but not on sperm cells (Blake, 1976; Blake and Sensabaugh, 1976). The minimum quantity of semen required for typing is about 50 $\mu\ell$ compared with about 5 $\mu\ell$ whole blood, since the concentration of Tf is 10 times lower in semen (Blake and Sensabaugh, 1978). Tf typing might, therefore, be possible in seminal stains under favorable conditions.

42.6 The Distribution of Tf Phenotypes in U.S. Populations

The data for American populations which have been studied are given in Table 42.1.

Table 42.1 Distribution of Tf Phenotypes in U.S. Population

Population	Total	Frequency — Number (Percent) C	B_1 C	B_2C	CD_1	Note	Reference
CAUCASIAN							
Southeastern GA	107	103(96.3)		1(0.9)	2(1.9)	1	Cooper et al., 1963
Southeastern US	2,221	2,194(98.8)	2(0.09)	15(0.68)	10(0.45)		Roop et al., 1968
Chicago, IL	101	101(100)					Shih and Hsia, 1969
Tecumseh, MI	7,654	7,560(98.8)		83(1.1)	11(0.14)		Schreffler et al., 1971
Greater Philadelphia, PA	203	202(99.5)			1(0.5)	2	Pakstis et al., 1978
	196	193(98.5)		2(1.0)	1(0.5)		
California	801	786(98.1)				3	Grunbaum et al., 1980
NEGRO							
New York, NY	99	89(89.9)			9(9.1)	4	Parker and Bearn, 1961a
Southeastern GA	133	120(90.2)			13(9.8)		Cooper et al., 1963
Southeastern US	418	399(95.5)			19(4.5)		Roop et al., 1968
Chicago, IL	101	93(92.1)			8(7.9)		Shih and Hsia, 1969
Greater Philadelphia, PA	164	151(92.1)			13(7.9)	2	Pakstis et al., 1978
	169	158(93.5)			10(5.9)		
California	502	467(93)				3	Grunbaum et al., 1980
OTHER							
New York, NY Chinese	116	109(94)				5	Parker and Bearn, 1961a
California and Mexico City Mexican	765	742(97)				3	Grunbaum et al., 1980
California and Hawaii Asians	1,295	1,259(97.2)					

1. One was B_{1-2} B_2
2. The sampling area included sections of PA and southern NJ. The two populations listed are separate sums of two members of twin pairs. Among the 169 members of the Black co-twin 2 population, one was D_1.
3. The following numbers (percents) listed as "rare": Caucasian 15(1.87); Negro 35(6.97); Mexican 23(3.01); Asian 36 (2.78).
4. One was D_1
5. 7 were CD_{Chi}.

SECTION 43. α_1–ANTITRYPSIN (Pi)

43.1 Introduction

The principal protease inhibitors in human plasma are α_1-antitrypsin (α_1AT), α_2-macroglobulin (α_2M), inter-α-trypsin inhibitor (IαI), antichymotrypsin (Achy; α_1X), antithrombin III (AT III) and C1 activator (c1-Ina) (Laurell and Jeppsson, 1975). The most important of these are α_1AT and α_2M, and α_1AT is the subject of this section because of the extensive genetic polymorphism it exhibits.

Proteolytic enzyme inhibiting activity in blood was recognized many years ago (Hahn, 1897; Camus and Gley, 1897). The complexity of the proteinase inhibitor system of plasma became more clear with the more recent studies of Grob (1943), Shulman (1952) and Jacobsson (1955). Shulman (1952) was able to differentiate plasmin inhibition activity from the inhibition of trypsin or chymotrypsin. Jakobsson (1955) recognized two trypsin inhibiting activities in plasma, one associated with α_2 globulins and the other with α_1 globulins. The latter was much more plentiful. The α_1 globulin associated trypsin inhibitory activity was isolated by Schultze *et al.* in 1955, but it was not recognized as such by this group until later (Schultze *et al.*, 1962c), and was called "3.5S α_1 glycoprotein". It has also been studied by Poulik and Smithies (1958), who called it α_1-globulin, Norman (1958) who called it α_1-antiplasmin, Bundy and Mehl (1959) who called it α_1-trypsin inhibitor, and Burtin (1964) who called it α_{1_A}-globulin. Schultze *et al.* (1962c) named the protein "α_1-anti-trypsin", and it is this designation that has persisted in the literature. The biochemical journals tend to call it α_1-protease inhibitor. Nomenclature is still something of a problem with this system, despite efforts to standardize it (see further below).

43.2 Genetic Variation

43.2.1 Multiple alleles controlling α_1-antitrypsin

In 1963, Laurell and Eriksson noted that the sera of several patients suffering from pulmonary degenerative diseases exhibited markedly reduced concentrations of α_1-antitrypsin, and suggested that there was a correlation between the two things. The possibility of a genetically controlled 'inborn error of metabolism' was raised. Eriksson (1964) showed that the pathological condition was hereditary, behaving like a recessive, and that homozygotes for the trait seemed to be most likely to have the emphysema. Family members presumed to be heterozygous had roughly half the normal α_1-AT levels in their serum. These observations were confirmed independently by Kueppers *et al.* (1964) and others. Eriksson and Laurell (1963) found an electrophoretically slow α_1-AT band, accompanied by the usual band, in the serum of a patient. The serum concentration was normal, and each band accounted for about half of it. The α_1-antitrypsins represented by the two bands were immunologically indistinguishable. The patient's three children had normal α_1-AT patterns. When a similar case was found (Axelsson and Laurell, 1965), genetic and family studies indicated not only that the slow α_1-AT variant was genetically determined, but that the gene for it was allelic to that for the previously observed α_1-AT deficiency.

In 1965, Fagerhol and Braend discovered a system of protein zones in the "prealbumin" region, which exhibited observable variation in low pH (4.95) starch gels. Five phenotypes were seen in the sera of 390 healthy Norwegian blood donors, and family studies showed that the system was inherited, and under the control of three alleles. The name "Pr proteins" was suggested for the system. The locus was designated *Pr*, and the alleles Pr^F, Pr^M and Pr^S (for "fast", "medium" and "slow"). The phenotypes were called MM, MS, SS, FM and FS. It was soon realized that the "Pr system" proteins were not "prealbumins", but that they corresponded to the α_1-AT proteins (Fagerhol and Braend, 1966; Kueppers and Bearn, 1966b), and Fagerhol and Laurell (1967) suggested that, since these proteins represented the major plasma proteinase inhibitors, the locus controlling their variation should be called *Pi* (for *P*rotease *i*nhibitor). The system exhibited a remarkable degree of genetic polymorphism. By 1968, Fagerhol and Tenfjord could list seven alleles at *Pi*. Arranged in order of decreasing electrophoretic mobility of their products on acid starch gels, these are Pi^F, Pi^I, Pi^M, Pi^S, Pi^V, Pi^X and Pi^Z. A full description was given by Fagerhol (1968). An additional phenotype, MW, accounted for by a new allele Pi^W, was found in two Spaniards by Fagerhol and Tenfjord (1968). The slow moving variant originally described by Eriksson and Laurell (1963) and Axelsson and Laurell (1965) corresponds to the product of Pi^X, while the gene for α_1-AT deficiency (homozygous serum having about 10–15% of the normal α_1-AT concentration) is Pi^Z. Descriptions of the alleles Pi^I, Pi^F, Pi^S and Pi^V may be found in Fagerhol (1967) and Fagerhol and Gedde-Dahl (1969). The latter study also included family studies confirming the codominant mode of inheritance of the various *Pi* alleles. Similar family studies were done by Kellerman and Walter (1970).

A number of additional phenotypes and variants have been described. Some are better characterized than others, and family data are apparently not available for all of them. The ninth allele, Pi^P, detected as MP, and of low frequency, was described by Fagerhol and Hauge (1968). Pi^E and Pi^G were described by Fagerhol at an international meeting in 1971 (discussed by Cox and Celhoffer, 1974). Pi MW^2 was described by Gedde-Dahl *et al.* (1972), MY^2 and ZY^2 by Porter *et al.* (1972), Pi^B seen as BM by Martin *et al.* (1973), and Pi^L in a Bantu population by Vandeville *et al.* (1974).

Cox and Celhoffer (1974) described Pi^N, seen as MN, and they reviewed the variants known up to that time.

43.2.2 Quantitative variation and complete deficiency

Several of the *Pi* alleles appear to be associated with reduced α_1-AT synthesis, in addition to coding for proteins that are structural variants. These are Pi^Z, Pi^P and Pi^S. Association of deficient allele phenotypes with diseases is discussed further below. In 1969, Kueppers found that the Pi^Z allele yielded a product electrophoretically distinguishable from that of the common Pi^M, and that MZ and ZZ people could be distinguished in this way. The frequency of this allele was about 7% in 100 random blood donors from California. Talamo *et al.* (1973) found a 24 year old man with advanced pulmonary emphysema whose serum had no detectable α_1-AT by any technique. Members of his family had half the normal activity. The total deficiency phenotype was called "Pi-", and the man was considered a homozygote for a Pi^- allele. The half activity family members were thought to be Pi^MPi^-, and their phenotypes were written "M/-" or "M-".

43.2.3 Refinements of Pi typing methods, isoelectric focusing, Pi M subtypes and other variants

A number of the Pi phenotypes can be detected by acid starch gel electrophoresis. Unequivocal characterization of some of the variants requires two dimensional crossed immunoelectrophoresis. Electrophoresis in the first direction is carried out in acid starch gels, and that in the second direction is done in antibody-containing gels at 90° to the first direction. Up to eight zones can be detected by the crossed immunoelectrophoresis technique, and the patterns differ according to phenotype. Agarose gel electrophoresis can be used for Pi typing as well, with protein staining or immunofixation for detection (Laurell and Persson, 1973; Jeppsson *et al.*, 1979). A standard must be run alongside unknown samples if simple protein staining is used to detect Pi zones (Laurell and Persson, 1973).

Application of isoelectrofocusing to Pi typing has shown that it provides a somewhat easier method for phenotyping some of the variants than two-dimensional immunoelectrophoresis, and it has further revealed some additional genetic variations in the Pi system. Procedures for Pi typing by isoelectric focusing on polyacrylamide gels have been described by Allen *et al.* (1974), Arnaud *et al.* (1975 and 1977) and Kueppers (1976b). Detection has been carried out by protein staining, which may be accompanied by densitometric tracing, and by immunofixation techniques (Arnaud *et al.*, 1977). Immunofixation can be done in the gel itself, or by "printing" onto an overlaid cellulose acetate membrane strip. Hercz and Barton (1978) described a Schiff's reagent staining technique for α_1-AT in polyacrylamide gels following isoelectric focusing, which had the advantages of showing only the isoproteins of α_1-AT, and of giving some information about the carbohydrate content of the components. Some of the phenotypic patterns are improved by prior treatment of the samples with cysteine, and isoelectric focusing of the mixed disulfides (Pierce *et al.*, 1976).

Isoelectric focusing has revealed that the common phenotype on starch gels, Pi M, can be resolved into at least six subtypes, accounted for by three different Pi^M alleles which are comparatively well distributed in Europeans. The nomenclature of the new variants can be quite confusing to the nonspecialist, in spite of the efforts of an international group to standardize it (see below).

In 1976, Frants and Eriksson found three phenotypes within Pi M, which they called M_1, M_1M_2, and M_2. The alleles responsible were Pi^{M1} and Pi^{M2}. Another allele, Pi^{M3}, which was difficult to detect by conventional isoelectric focusing, was reported by Frants and Eriksson in 1978. The product of the Pi^{M3} allele could not be detected very well by conventional PAGIF when it was paired with another Pi^M allele, but could be distinguished when paired with a different *Pi* allele. However, the introduction of so-called "separators" into the gels along with the ampholines, a technique called "separation isoelectric focusing" or "SIEF", enabled the differentiation of the products of all three Pi^M alleles. The separators cause "plateaus" in the pH gradients, which can improve resolution in certain systems (Frants *et al.*, 1978). According to Frants and Eriksson (1978), the previously described Pi M_1 resolves to M_1, M_1M_2 and M_2, Pi M_1M_2 becomes M_1M_3, and Pi M_2 resolves to M_2M_3 and M_3 in the new three allele system. Kueppers (1976a) found one of these alleles, and called it Pi^{M1} where Pi^M was the usual allele. Van den Brock *et al.* (1976) found the same allele, and termed it Pi^{MN}. In 1978, Kueppers and Christopherson described the other allele. Klasen *et al.* (1977) also described the third common allele of Pi^M. Genz *et al.* (1977) found all six phenotypes, and they called the alleles Pi^{Ma}, Pi^{Mb} and Pi^{Mc}. The phenotypes were Pi Ma, Mb, Mc, Mab, Mac and Mbc. The equivalencies between these different designations, framed in terms of the Pi Committee recommendations, are: The Pi M_1, M_1M_2 and M_2 of Frants and Eriksson (1976) are equivalent to M, MM_1 and M_1 (Kueppers, 1976a), Ma, Mac and Mc (Genz *et al.*, 1977), and M, MM_N and M_N (van den Brock *et al.*, 1976). The committee recommended that these be called, respectively, M_1, M_1M_2 and M_2. The Pi^{M3} of Kueppers and Christopherson (1978) is apparently the same as that of Klasen *et al.* (1977) and of Frants and Eriksson (1978). The Pi^{M2} is also equivalent to the Pi^N of Constans and Viau (1975), but not to the "Pi^N" of Cox and Celhoffer (1974). The M_1, M_1M_2 and M_2 phenotypes have been studied in the Japanese population by Harada *et al.* (1977).

Some additional phenotyes reported in the Pi system are M_{Lamb} and $M_{Baldwin}$ (Johnson, 1976), Pi W3 Constantine (Khitri *et al.*, 1977), Pi Nhampton (or Nham) (Arnaud *et al.*, 1978a), Pi B Alhambra (Yoshida *et al.*, 1979), Pi T (Kühnl and Spielmann, 1979b and 1979c), Pi <L (Kühnl and Spielmann, 1979b) and Pi Gam (Welch *et al.*, 1980). There are quite a few others as well, some better characterized than others, and they are discussed in Cox *et al.* (1980).

It may be noted here that *Pi* has been shown to be linked to *Gm* (Gedde-Dahl *et al.*, 1972). Further discussion and additional data are given by Fagerhol (1976). Recombination frequency was dependent in certain ways upon the Pi alleles

present, and the phenomenon is apparently not yet fully understood.

43.2.4 Standardization of nomenclature and techniques

Pi phenotype identification is apparently not as easy to master nor as straightforward as that for many other systems, especially when variant phenotypes are involved. Some variants are more difficult to diagnose than others. Special isoelectrofocusing techniques are required to be certain about the identity of the Pi M_3 phenotypes. In 1978, an international committee met to consider the complexities of the Pi system, and their work is reported by Cox (1978) and Cox *et al.* (1980). The committee recommended that the protein be designated α_1-antitrypsin (α_1AT), and its genetic locus as *Pi*. Several reference laboratories have been established, and they try to maintain panels of variants against which "new" ones can be compared by various different techniques. In our part of the world, one reference laboratory is maintained at the Hospital for Sick Children in Toronto (Dr. Diane Wilson Cox), and another is at the University of North Carolina Medical Center in Chapel Hill (Dr. A. Myron Johnson). Details are published in Cox *et al.* (1980). The committee established rigorous standards for confirmation of variants as "new". They noted that several different techniques must be used to compare a "new" variant with known ones. In addition, they recommended some standardization of the nomenclature. A laboratory manual has been prepared under NIH sponsorship in this country (Talamo *et al.*, 1978), and it contains the detailed standard procedures. The papers should be read for the details. A few highlights are: The M1, M2 and M3 designations for the common subtypes of Pi M were retained, their alleles being Pi^{M1}, Pi^{M2} and Pi^{M3}; upper case letters will continue to be used to indicate relative position in isoelectric focusing gradients, and descriptive names can be appended as needed; lengthy descriptive names can be abbreviated by using the first few letters of the name, e.g. Pi $E_{Cincinnati}$ = Pi E_{Cin}, Pi $M_{chapelhill}$ = Pi M_{cha}, and so forth; the complete deficiency allele is called Pi^{Null}. A number of additional variants are discussed in Cox *et al.* (1980). It is also clear from the report that unequivocal diagnosis of some variant Pi types is not always an easy matter, even for experienced laboratories. They recommended against Pi typing in ordinary clinical laboratories, suggesting instead that a few reference laboratories be set up for the purpose, each serving a geographical area containing 5 to 10 million people. These larger labs would have the resources and volume of work to maintain complete sets of reference sera, and so forth.

Not everyone agrees completely with the committee's recommendations. Yoshida (1979) took exception to several of them. He noted that the name "alpha-antitrypsin" had become widely used by clinical workers, but that the biochemical journals still preferred "α_1 protease inhibitor". He preferred the latter, since the ". . . . .protein is not an antibody against trypsin", and because that name is compatible with the locus name, *Pi*. He said further that the guidelines for the identification and publication of new variants imposed unreasonable restrictions on investigators, and should not be adopted. Restricting the criteria to electrophoretic or electrofocusing mobilities was a mistake, he said, because variants not differing in charge would be missed. Biochemical properties, including kinetic properties, amino acid analyses, etc., should be included too, he said. It would also be helpful if standard electrophoretic and isoelectrofocusing procedures were recommended, and all established variants run in the standard systems, and the results published.

43.3 Relationship of α_1–Antitrypsin Deficiency and Disease

There is a well established connection between certain kinds of degenerative lung diseases and the Pi^Z allele. Homozygotes for the gene, Pi ZZ people, have a high risk of developing chronic obstructive pulmonary disease (COPD), which leads to emphysema. Similarly, Pi ZZ infants are at high risk for developing neonatal hepatitis and cirrhosis of the liver. This material is discussed by Kueppers and Bearn (1966a), Kueppers and Black (1974), Fagerhol (1976) and has been reviewed by Morse (1978). In homozygous deficient subjects (ZZ), and less so in Z-heterozygotes, some of the liver cells accumulate α_1-AT (see, for example, Lieberman *et al.*, 1972), and this leads to liver cell necrosis. Most investigators think that Pi SZ people are at higher risk for COPD as well, but there is some controversy about the risk of MZ or MS individuals. The pathogenesis of the lung disease almost certainly has nothing to do with trypsin, which rarely gets into circulation. α_1-Antitrypsin can inhibit other proteases, notably leucocyte collagenase and elastase. These enzymes have been implicated in the production of emphysema lesions, and their excess activity in α_1-AT deficient subjects may provide a basis for understanding the relationship. There is much more to it, and more work will be required before an understanding is achieved. For one thing, there is quite a bit of individual variation among ZZ people with respect to clinical manifestations in lung or liver. Another thing is that α_1-AT deficiency is implicated in only a small percentage of degenerative lung disease patients. There is some evidence of the association of α_1-AT deficient phenotypes with other diseases. Although the frequency of Pi^Z is significant in many populations, and 2 to 5% of some European and American populations may be considered "at risk" on the basis of the preliminary data, there is still a question as to whether mass screening programs are appropriate. These matters are discussed by Fagerhol (1976) and Morse (1978). Chapuis-Cellier and Arnaud (1979) recently reported that the Pi^Z allele is preferentially transmitted to offspring when it is present in the father suggesting that Pi^Z-carrying sperm enjoy a selective advantage of some sort.

43.4 Biochemical Studies

Some biochemical properties of α_1-AT were mentioned at the beginning of the section, in connection with the earlier studies on its isolation and characterization. More recent purification and isolation studies are reviewed by Laurell

and Jeppsson (1975). Schultze *et al.* (1963) described the purification procedures used at that time. α_1-Antitrypsin is an inhibitor of a number of proteolytic enzymes besides trypsin, including plasmin, chymotrypsin, thrombin (see Rimon *et al.*, 1966), elastases and collagenases. The esterase activity of these enzymes is inhibited as well. The mechanism of inhibition involves some kind of complex formation, but its exact nature has not been completely elucidated. α_1-AT is unstable at low pH (less than 5) and loses its inhibitory capacity. The activity can be recovered, however, by titrating back up (Glaser *et al.*, 1977). Aggregates form at about pH 4 which retain immunological identity, but which do not inhibit proteases. There is microheterogeneity associated with α_1-AT regardless of the phenotype, and its biochemical basis is not very clear. Musiani *et al.* (1976) isolated 8 fractions from Pi M sera on a preparative scale, and the fractions showed various distributions in a pH 4–6 isoelectric focusing gradient. These fractionation results will now have to be interpreted in terms of the now recognized Pi M subtypes. Hodges *et al.* (1979) isolated and characterized the oligosaccharide chains of the α_1-AT molecule (from Pi M people). There are two types of chains, a large one with a complicated structure, and a smaller one. It appeared that each protein contains one large and three small chains. Both kinds are attached to the peptide through GlcNAc—Asn linkages. The large chain can have up to three terminal N-acetyl-neuraminic acid residues, and the small chain has two. Therefore, there could be up to 9 terminal NANA residues in the intact molecule, if these relative amounts are correct.

There are a few biochemical studies on the protease inhibitor from specific different phenotype sera. Only in a few cases have the amino acid differences between variant and M molecules been worked out. The difference between Pi M and Pi S resides in a single amino acid (Owen *et al.*, 1976; Yoshida *et al.*, 1977). Pi S has Val where Pi M has Glu, and this was clear in two completely different examples of the protein. Jeppsson *et al.* (1978) purified Pi M, S and Z molecules, and said that they had detected a single amino acid difference in one cyanogen bromide peptide between M and Z. M had Glu where Z has Lys. Yoshida *et al.* (1979) characterized their Pi B Alhambra mutant fully, and found two amino acid substitutions, Lys→Asp and Glu→Asp. Hercz and Barton (1977) have purified Z protein, and found that it contained more glycine residues than the M protein, but that it did not differ in carbohydrate content. They also discussed some of the apparently conflicting results of various workers. Bauer *et al.* (1978) have isolated and studied Z protein material from the liver of a Pi ZZ individual. It was homogeneous, and antigenically intact, but had no proteinase inhibitor activity. There was no galactose or sialic acid in the carbohydrate moiety and GlcNAc was reduced. Mannose was present in excess, however.

Arnaud *et al.* (1978b) studied a number of individuals who had the Pi^{l} allele. It was found that this allele is in the class of "deficient" alleles, in that people who had it showed reduced protease inhibitor levels. Further, two subtypes of Pi^{l}, called I_1 and I_2, were detected by electrofocusing. Kueppers *et al.* (1977) described a woman with a Pi M phenotype, but who was deficient in α_1-AT. She had emphysema, and her father had COPD. He had normal levels of α_1-AT, but her mother and two sibs had about 50% normal levels. This family may have a variant "M", which is indistinguishable from the usual M, and the possibility of Pi^{Null} cannot be ruled out in the family. Kahn *et al.* (1977) conducted studies on a number of normal, partially deficient and fully deficient individuals, and the results indicated that α_1-AT contributes >90% of the total antitrypsin activity of normal plasma.

Reviews of the Pi system may be found in Fagerhol (1968 and 1976), Giblett (1969), Fagerhol and Laurell (1970) and Laurell and Jeppsson (1975).

43.5 Medicolegal Applications

No direct references to the application of the Pi system to disputed parentage cases were found in the literature, although a number of authors mention its obvious applicability. The system is much more powerful if Pi M subtyping is performed, and specialized laboratories will probably be employing the system in this way.

There has been little reported work on Pi phenotyping in bloodstains. Frants and Eriksson (1979) indicated that they had obtained promising preliminary results on Pi phenotyping in dried blood by isoelectrofocusing. The system would be most useful if Pi M subtypes could be determined. As was noted above, difficulties can be encountered with many of the variant phenotypes. Stored sera tend to develop "storage" bands, which make the already complex patterns more difficult to interpret, but sulfhydryl reagents alleviate this problem appreciably. A study of the ability to determine Pi phenotypes in postmortem bloods was done by Conrad *et al.* (1979). Sera stored frozen (−20°) retained their phenotypic characteristics quite well if the pH was 7.0–7.5. 86% of the sera stored for 2–2½ years, but only 30% of those stored for more than 4 years, could be typed. There was some decrease in the typability of sera that were not collected immediately after death.

α_1-AT is found in genital secretions as well, where it is suspected of having some role in fertilization (Hirschhäuser *et al.*, 1972), and it is possible that the protein might provide a genetic marker in these fluids. The cervical mucus shows cycle-dependent variations in α_1-AT content, which are affected by oral contraceptive therapy (Schumacher, 1970). α_1-AT is found in semen at mean levels of 68–87 $\mu g/m\ell$, but not in sperm cells (Schumacher, 1970; Blake, 1976; Blake and Sensabaugh, 1976 and 1978). Blake and Sensabaugh (1978) estimated that about 100 $\mu\ell$ semen would be needed to detect α_1-AT, compared with about 5 $\mu\ell$ whole blood. They did not regard it as a very good prospect for typing in forensic samples containing semen. It is not completely clear whether the Pi phenotypes observed in serum are expressed in semen in the same way.

43.6 Distribution of Pi Phenotypes

There have been very few studies in U.S. populations, and in some cases only the gene frequencies were reported. Leaving aside Pi M subtyping for a moment, it is found that Pi M is the common phenotype in most populations examined. In Europeans, the Pi^S frequency is polymorphic, and is higher in Spain, Portugal and the south of France than elsewhere. The frequency of FM varies from about 0.2 to about 2%, and that of MZ is between about 0.2 and 5%. Pi^Z is more frequent in Caucasians, and relatively few non-M phenotypes seem to occur in Africans, Asians, Finns, Lapps and Greenland Eskimos.

In St. Louis, MO, Dew *et al.* (1973) reported $Pi^M = 0.9480$, $Pi^F = 0.0027$, $Pi^I = 0.0012$, $Pi^S = 0.0344$ and $Pi^Z = 0.0127$ in 2,047 Caucasians. In another St. Louis study, 1,933 Caucasians were 90% M, 6.7% MS and 2% MZ, while 204 Negroes were 96.1% M, 2% MS and 1% MZ (Pierce *et al.*, 1975). The gene frequencies observed in 188 Caucasians in San Francisco were very similar to those in St. Louis (Kueppers, 1971). Kueppers and Christopherson (1978) reported the frequencies of Pi phenotypes, including subtypes, in 240 Whites (Rochester, MN and southeastern PA), and in 304 Blacks (southeastern GA and Philadelphia, PA). Among the Caucasians were: 98 (41%) M_1, 14 (5.8%) M_2, 2 (0.8%) M_3, 51 (21.3%) M_1M_2, 39 (16%) M_1M_3, 7 (2.9%) M_2M_3, 1 (0.4%) FM_1, 2 (0.8%) IM_1, 12 (5%) M_1S, 6 (2.5%) M_2S, 2 (0.8%) M_3S and 6 (2.5%) M_1Z. Among the Negroes were: 248 (81.6%) M_1, 1 (0.3%) M_3, 15 (4.9%) M_1M_2, 29 (9.5%) M_1M_3, 2 (0.7%) M_2M_3, 1 (0.3%) IM_1, 3 (1%) FM_1, 3 (1%) M_1S and 2 (0.7%) M_1Z. The Pi^{M1}, Pi^{M2} and Pi^{M3} allele frequencies were, respectively, 0.64, 0.19 and 0.11 in Whites, and 0.903, 0.028 and 0.054 in Blacks.

SECTION 44. GENETIC MARKERS OF THE IMMUNOGLOBULINS—Gm, Km, Am AND Hv

44.1 Introduction

The genetically determined antigenic markers which comprise the Gm and Km (and the Am and Hv) systems are located on the immunoglobulins, the family of serum proteins which possess antibody activity. The classes and structure of the immunoglobulins were discussed in section 1.3.3.2. There are five classes of these molecules, each possessing a different type of heavy chain. Immunoglobulins have the general structure H_2L_2, where H stands for a heavy chain and L for a light chain. There are two types of light chains, κ and λ, both of which occur in individuals, but not in the same molecule. IgM is a polymer of the basic H_2L_2 structure.

Gm and Km are among the most complicated of the genetic marker systems, and they are very useful in forensic problems. Among the many excellent reviews of these systems are Prokop and Bundschuh (1963), Natvig and Kunkel (1968), Franklin and Fudenberg (1969), Giblett (1969), Görtz (1969), Grubb (1970), Mage *et al.* (1973), Natvig and Kunkel (1973), Ropartz (1974) and Giblett (1977).

44.2 Genetic Variation in the γ Chains of IgG—The Gm System

Some terminology arises in discussions of Gm that is not commonly used in most of the other systems, and it will be introduced briefly here. Three degrees of specificity related to structural variation have been differentiated, based on the antigenic properties of the immunoglobulin molecules within a given species (Giblett, 1969). *Isotypic* specificities are common to all members of a species, but differentiate immunoglobulin molecules into classes and subclasses. *Allotypic* specificities differ within a species, and reflect inherited variation of molecular structure. The polymorphic genetic marker systems (Gm, Km) represent allotypic specificity differences, and one can and does refer to the "Gm allotypes". *Idiotypic* specificities are characteristic of the products of single cells (or their clones). Myeloma proteins are a good example. The term *haptotype* is not used too much in discussions of Gm genetics, but it could be. It is a very common term in HLA genetics. When a chromosome segment carries closely linked genes that are part of the same system, and which tend to be inherited together as a unit, the gene makeup of one or the other homologous chromosome segment is called a *haplotype*. In the Fisher-Race conception of Rh genetics, therefore, one could speak of Rh haplotypes. Similarly, one could use the term in MNSs and in Gm genetics, though it seems to be encountered most often in discussions of HLA.

44.2.1 The Gm factors

In 1956, Grubb found that Rh+ cells sensitized with some examples of incomplete anti-D were agglutinated by the sera of some patients who had rheumatoid arthritis. Further, this agglutination could be inhibited by the sera of some 60% of normal random donors. Similar observations were made by Milgrom *et al.* (1956) and by Waller and Vaughn (1956). The inhibiting property of normal serum was quickly shown by Grubb and Laurell (1956) to be inherited as a simple Mendelian dominant. The responsible factor was found in the gamma globulin fraction (now known as IgG), and it was called Gm(a). The active rheumatic sera contained an "anti-Gm(a)" which agglutinated anti-D sensitized Rh+ cells, provided the anti-D IgG possessed the Gm(a) determinant. Normal sera having Gm(a) on the IgG molecules reacted with the anti-Gm(a) in the active rheumatic sera, thus inhibiting its ability to agglutinate the sensitized Rh+ cells. Soon afterward, two other genetically determined, serologically detectable differences in the IgG were found using different reagent pairs (rheumatoid serum agglutinator and sensitizing anti-D), and they were called Gm(c) (Harboe and Lundevall, 1959) and Gm(b) (Harboe, 1959).

By 1965, approximately 14 distinct Gm specificities had been identified, and nomenclature had become something of a problem. A great deal of new information had also become available about the structure and properties of immunoglobulins around this time. In 1964, the nomenclature of human immunoglobulins was standardized (W.H.O., 1964). As a logical outgrowth of that work, a standardized nomenclature for the Gm and Km allotypes was recommended in the following year (W.H.O., 1965; Ceppellini and many others, 1966). Numbers were assigned to the Gm and Km specificities, and their use was recommended instead of the letters that had been used. In the more recent nomenclature revision, however, letter designations and their numerical equivalents are apparently equally acceptable (W.H.O., 1976). More will be said about the recent recommendations in appropriate sections below.

Almost 30 different Gm factors have been described. Some have been found to be the same as others, and in some cases, the specificities can no longer be tested because there are no longer any reagents available. In a few cases, the relationships of particular factors to the rest of the system are not very clear. The list of factors, and some of their equivalent names, is shown in Table 44.1.

The original anti-Gm antibody, now called anti-Gm(a), was found in the serum of a rheumatoid arthiritis patient, and many of the subsequent examples of antibodies defining

Table 44.1 Genetic Markers of the Immunoglobulins

Chain Location	Recommended Designation: Alphameric	Recommended Designation: Numeric	Other or Previous Designations: Alphameric	Other or Previous Designations: Numeric	Reference(s)
Allotypic Markers of the γ Chains					
IgG1:	G1m(a)	G1m(1)	Gm(a)		Grubb, 1956
	G1m(x)	G1m(2)	Gm(x)		Herboe and Lundevall, 1959
	G1m(f)	G1m(3)	Gm(b^w), (b2), (f)	Gm(3),(4)	Steinberg and Wilson, 1963; Gold et al., 1965; Steinberg, 1965
	G1m(z)	G1m(17)	Gm(z)	Gm(17)	Litwin and Kunkel, 1966a and 1966b
IgG2:	G2m(n)	G2m(23)	Gm(n)	Gm(23)	Kunkel et al., 1966; Natvig and Kunkel, 1967
IgG3:	G3m(b0)	G3m(11)	Gm(b^β), (b0)	Gm(11)	Ropartz et al., 1963; van Loghem and Martensson, 1967
	G3m(b1)	G3m(5)	Gm(b), (b1), (jb^γ)	Gm(5),(12)	Harboe, 1959; Ropartz et al., 1963; van Loghem and Martensson, 1967
	G3m(b3)	G3m(13)	Gm(b3), (Bet)	Gm(13),(25)	Steinberg and Goldblum, 1965
	G3m(b4)	G3m(14)	Gm(b4)	Gm(14)	Steinberg and Goldblum, 1965
	G3m(b5)	G3m(10)	Gm(b^a), (b5)	Gm(10)	Ropartz et al., 1963; van Loghem and Martensson, 1967
	G3m(c3)	G3m(6)	Gm-like, (c), (c3)	Gm(6)	Steinberg et al., 1960
	G3m(c5)	G3m(24)	Gm-like, (c), (c5)	Gm(24)	van Loghem and Martensson, 1967
	G3m(g)	G3m(21)	Gm(g)	Gm(21)	Natvig, 1968
	G3m(s)	G3m(15)	Gm(s)	Gm(15)	Martensson et al., 1966
	G3m(t)	G3m(16)	Gm(t)	Gm(16)	Martensson et al., 1966
	G3m(u)	G3m(26)	Gm(Pa)		van Loghem and Grobbelaar, 1971; Steinberg, 1977
	G3m(v)	G3m(27)	Gm(Ray)		
		G3m(28)		Gm(28)	Rivat et al., 1978
Markers whose status is unclear and/or for which reagents are no longer available					
–			Gm(r)	Gm(7)	Brandtzaeg et al., 1961
–			Gm(e)	Gm(8)	Ropartz et al., 1962
IgG1			Gm(p)	Gm(9)	Waller et al., 1963
IgG1			RO2, Rouen 2	Gm(18)	Ropartz et al., 1967
–			RO3, Rouen 3	Gm(19)	Ropartz et al., 1966
–			San Francisco 2	Gm(20)	Klemperer et al., 1966; Ropartz et al., 1966
–			Gm(y)	Gm(22)★	Litwin and Kunkel, 1967
Light Chain Markers					
κ	–	Km(1)	InV, Inv(1)(f)	Inv(1)	Ropartz et al., 1962
	–	Km(2)	Inv(a)	Inv(2)	Ropartz et al. 1961a and 1961b
	–	Km(3)	Inv(b)	Inv(3)	Steinberg et al., 1962
Isoallotypic Markers (the so-called "non-markers")					
	nG1m(a)	nG1m(1)	non-a		Natvig et al., 1969
	nG1m(z)	nG1m(17)	nG1m(z)		
	nG3m(b0)	nG3m(11)	non-b0		Gaardner and Natvig, 1972
	nG3m(b1)	nG3m(5)	non-b1		Gaardner and Natvig, 1972
	nG3m(g)	nG3m(21)	non-g		Natvig et al., 1969
	nG4m(a)		4a		Kunkel et al., 1970
	nG4m(b)		4b		Kunkel et al., 1970
		nA2m(2)		nA2m(2)	
Other Markers					
IgA2:	A2m(1)			$Am(1), Am_2$	Vyas and Funderberg, 1969
	A2m(2)			$A_2M(2)$	van Loghem et al., 1973
IgG1				Isf(1)	Ropartz et al., 1966 and 1968
Heavy chains (variable region) of IgG, IgM and IgA				Hv(1)	Wang et al., 1978

Recommended nomenclature after W.H.O. (1976)

★ Equivalent to nG1m(a) = nG1m(1) (Ropartz, 1974)

Gm specificities have been found in such sera. These are called "Ragg" antibodies (for *R*heumatoid *Agg*lutinating). Examples of various anti-Gm of various specificities are also found in non-rheumatoid sera (Ropartz *et al.*, 1960), especially in multiply transfused patients, women who have been pregnant, and in children. These antibodies are called "SNagg" (for *S*erum *N*ormal *Agg*lutinating). The former are usually quite potent, but may have autoantibody activity, multiple specificity and they often show prozones. The latter are usually of lower titer, but they are often monospecific and do not show prozones. They are the preferred reagents for Gm typing.

Gm(1), or Gm(a), was the first specificity to be described (Grubb, 1956) as noted above. Antisera to Gm(1) have been found in several hundred people. Hemagglutination inhibition remains the best typing method, and the anti-D must come from a Gm(1) person, preferably someone containing as few other Gm factors as possible. It has occasionally been reported that Gm(1) can be detected by one reagent system but not another, and there are reports of reagent systems that appear to differ in the detection of Gm(1) in different racial groups. Gm(2), or Gm(x) was described by Harboe and Lundevall (1959), and many additional examples of anti-Gm(2) from rheumatoid and normal sera have been found. Gm(2) is rare in Black populations. Gm(3) was defined by the serum of a 4 year old Black boy (Steinberg and Wilson, 1963), and was called Gm(b^w). It has also been called Gm(b^2) (Steinberg and Goldblum, 1965). Anti-Gm(3) has not been found in anyone else (Grubb, 1970). Gm(4) was described by Gold *et al.* (1965) who called it Gm(f). Examples of anti-Gm(4) have been found in more normal people than rheumatoid arthritis patients. Discrepant results have been reported in Gm(4) detecting systems, depending upon the reagents used. There is considerable evidence to suggest that Gm(4) is not different from Gm(3) (Steinberg, 1965), and they are regarded as identical. Gm(5) was described by Harboe (1959) who called it Gm(b). It has been called Gm(b^1) (Steinberg and Goldblum, 1965), and Gm(b^γ). Anti-Gm(5) antibodies have been found on a number of occasions. Those which readily distinguish Gm(5) from Gm(−5) in Caucasians can give markedly different reactions when used in other populations. This discovery has led to the delineation of a number of additional Gm factors. Gm(6) was first called "Gm-like" by Steinberg *et al.* (1960), and later Gm(c) (Steinberg and Wilson, 1963). It has also been called Gm(c5). A number of examples of anti-Gm(6) have been found. Most of the antibodies have come from Whites, many with rheumatoid arthritis, although the factor is mostly found in Blacks. Gm(7) was described by Brandtzaeg *et al.* (1961), and called Gm(r). Another anti-Gm(7) has been found, but apparently most of the reagents have now been exhausted. Gm(8) was first described by Ropartz *et al.* (1962), and then named Gm(e). The original anti-Gm(8) is the only one that had been found up to 1970, and it came from a rheumatoid arthritis patient. Gm(9) was originally called Gm(p) (Waller *et al.*, 1963), and two examples of anti-Gm(9), both from patients with rheumatoid arthritis, were described. Further examples had not been found by 1970. Gm(10) was described by Ropartz *et al.* (1963) as Gm(b^α). The anti-Gm(10) came from a healthy Caucasian, and no further examples had been seen by 1970. Grubb (1970) said that further studies would be needed to show that Gm(10) was different from Gm(13). Indeed, Johnson *et al.* (1977) regarded Gm(10) and Gm(13) as identical. Gm(11) and Gm(12) were also found by Ropartz *et al.* (1963), and called Gm(b^β) and Gm(b^γ), respectively. Gm(11) has been called Gm(b^0) (van Loghem and Martensson, 1967). Other examples of anti-Gm(11) have been found. Gm(11) and Gm(5) are usually found together in Caucasians. Gm(12) is not clearly distinguishable from Gm(5), and they are considered identical. Gm(13) was the Gm(b^3) of Steinberg and Goldblum (1965). It has also been called Gm(Bet). Further anti-Gm(13) sera have been found. As noted above, Grubb (1970) said that Gm(13) and Gm(10) need to be more clearly distinguished. Gm(14) is the Gm(b^4) of Steinberg and Goldblum (1965). Anti-Gm(14) sera are apparently rare. Gm(15) and Gm(16) were described as Gm(s) and Gm(t) by Martensson *et al.* (1966). Some further examples of antisera to both have been found. Gm(17) is the Gm(z) of Litwin and Kunkel (1966a and 1966b). It was detected by a rabbit anti-Gm(17) prepared by immunization with the Fab fragment of IgG1 myeloma protein, followed by absorption with Gm(−17) immunoglobulin. Gm(18) is the Gm (Ro2), or 'Rouen-2' of Ropartz *et al.* (1967). Antisera are apparently very rare. Gm(19) is Gm(Ro3) or 'Rouen-3' of Ropartz *et al.* (1966). The antiserum was difficult to work with. Gm(20) was described by Klemperer *et al.* (1966), defined by an antibody from a rheumatoid arthritis patient. No further examples have been found. Gm(20) was only found in Gm(1) people. Gm(21) is the Gm(g) of Natvig (1966). The first antisera were human Raggs, but anti-Gm(21) has been produced in animals. Gm(21) is almost always reciprocally related to Gm(5) and Gm(11). Gm(22) is the Gm(y) of Litwin and Kunkel (1967), and was defined by a rabbit anti-IgG1 (Gm−1,4) myeloma protein. Anti-Gm(22) are apparently very scarce, if there is still any available at all. Gm(23) is Gm(n) (Kunkel *et al.*, 1966), and was detected by an antiserum prepared in primates against IgG2 myeloma protein. Gm(23) is the only allotypic marker known on IgG2. Gm(23) can be detected by precipitation in gels, the first Gm specificity to be detected in this way. The technique requires too much precious antiserum, however. No anti-D with Gm(23) has ever been found, so a different technique was used (Natvig and Kunkel, 1967). IgG2 myeloma protein with Gm(23) can be coated onto red cells using bis-diazotized benzidine, thus providing the "coat" for the test system. Tanned red cells did not work very well (see sections 1.3.4.1 and 16.3). Gm(24) is apparently the Gm(c^5) of van Loghem and Martensson (1967). Gm(25) appears to be identical with Gm(13) and no longer treated separately. Thus, as noted above in connection with Gm(10), Johnson *et al.* (1977) regard Gm(10) = Gm(13) = Gm(25). Gm(26) was described by van Loghem and Grobbelaar (1971) on the basis

of immune sera prepared in baboons. Steinberg (1977) described further studies on it. Gm(26) is also called Gm(u), and has been called Gm(Pa). Gm(27) is known as Gm(v) and Gm(Ray). Gm(28) was recently described by Rivat *et al.* (1978). A Gm factor called "L1" was described by Blanc *et al.* (1976) in two families, one French and the other Algerian. It was transmitted with Gm(1) and Gm(17), was always present in Gm(21) sera, and always absent in Gm(5) sera. It is unclear whether it has yet been assigned a number. Ropartz (1974) said that Gm(22) or Gm(y) had been found to be equivalent to Gm(non-a), which according to the nomenclature recommendations should be designated nGlm(1) or nGlm(a) (see further below).

44.2.2 Assignment of Gm factors to IgG subclasses

Existence of isotypic subclasses of IgG has been recognized for some time. In certain neoplastic diseases, especially multiple myeloma, immunoglobulins are synthesized in excess. Urine may contain an unusual protein in these conditions, now known to represent immunoglobulin light chains, and called Bence-Jones protein. The IgG synthesized in multiple myeloma is unique in its high degree of homogeneity, as compared with the normal complex mixture of these molecules. The homogeneity of the myeloma protein is attributable to their synthesis by a highly selected population of cells, rather than by the sum total of all immuno-globulin-synthesizing cells. The neoplastic cells producing myeloma proteins are regarded as being derived from a single clone. Because of the homogeneity of myeloma proteins, and the fact that they can be obtained in large amounts, they are very important tools for immunochemists and immunologists. In 1964, Grey and Kunkel made rabbit antisera to various myeloma proteins, and absorbed them with other myeloma proteins. They were able by subsequent immunodiffusion tests to distinguish four subclasses of IgG, which they called We, Ne, Vi and Ge. Other investigators obtained similar results independently, but used different names for the subclasses. We now call the subclasses IgG1, IgG2, IgG3 and IgG4, according to the W.H.O. recommendations. The equivalent older names are IgG1 = We, γ2b, C; IgG2 = Ne, γ2a; IgG3 = Vi, γ2c, Z; and IgG4 = Ge, γ2d. All four subclasses are present in normal sera, though not in equal concentrations. The antigenic determinants which characterize them are, therefore, isotypic. There is considerable structural similarity in the peptides derived from the four subclasses (Grey and Kunkel, 1967).

Through studies of IgG myeloma proteins for both subclass and Gm specificity, the Gm antigens have been assigned to the various subclasses of IgG on which they reside. These assignments are now reflected in the formal nomenclature (W.H.O., 1976). Those antigens residing on IgG1 are denoted "Glm(1)", "Glm(2)", etc. Those on IgG3 are called "G3m(11)", "G3m(5)", etc. The letter designations may be used as well, as indicated in Table 44.1. Gm(23) or Gm(n) is formally designated G2m(23) or G2m(n), and is the only known Gm specificity residing on IgG2. This nomenclature is extended to cover genetic markers on other immunoglobulin heavy chains as well (see below).

Each subclass of IgG is thought to be under the control of a separate genetic locus. Thus, if genes determining two or more Gm factors are present on a single chromosome, they may be expressed together, separately, or not at all, on a given IgG molecule. It will depend on the subclass of that molecule. It may also be noted that myeloma proteins do not possess the same phenotype as serum from the same person. In any given plasma cell, only one of the paired genes for immunoglobulin synthesis on homologous chromosomes is active. Immunoglobulins provide the only known example of autosomal allele exclusion in humans, i.e., only one allele is active in a given cell. This phenomenon apparently also occurs with the entire X chromosome in females (see sections 1.2.4.4 and 33.1.4). In a Gm(1,3,5) Caucasian, for example, IgG myeloma proteins contain only one, or none, of these determinants. The reason is that Gm(1) and Gm(3) are at different locations in IgG1, and the genes are on opposite homologous chromosomes, while that for Gm(5) is in IgG3.

44.2.3 Isoallotypic markers of immunoglobulins—The nonmarkers

Because myeloma proteins have been extensively used in the study of immunoglobulin markers, it has been possible to define a new class of genetic marker. Myeloma proteins represent only one subclass of IgG, and as noted above, correlation studies on these proteins of subclass and Gm antigens have permitted assignment of Gm factors to particular subclasses. Antigens have been discovered which are shared by different subclasses, but which exhibit genetic variation in only one of them. The immunoglobulin system thus shows two classes of markers. In one, a genetic event is related to a subclass-specific region, and two regular allelic genes control a pair of markers, like Glm(3) and Glm(17). In the other, a genetic event is shared by other subclasses, reflecting the structural homologies preserved in subclass genes. Here, one may have a regular Gm marker behaving as an allele in one subclass, like Glm(1), but where the antithetical marker is shared by other subclasses. These latter are referred to as "nonmarkers" by Natvig and Kunkel (1973), and are called "isoallotypic markers" by the W.H.O. Nomenclature Committee. The human nonmarkers were detected by immunization of animals with myeloma proteins reflecting a particular subclass. The first was "non-a" (Frangione *et al.*, 1966; Natvig *et al.*, 1969). Both the peptide and the antigenic marker were present in all Glm(−1) IgG1 proteins, and in all IgG2 and IgG3 regardless of type, but absent in IgG4. It is recommended by the W.H.O. Nomenclature Committee that "non-a" be designated "nGlm(1)" or "nGlm(a)", as indicated in Table 44.1. There are several other nonmarkers as well. "Non-g" was found in G3m(g) [G3m(21)]-negative IgG3 proteins,

and in all IgG2 (Natvig *et al.*, 1969). Antigens "non-b0" and "non-b1" [nG3m(b0) and nG3m(b1)] are present on IgG3 molecules which lack G3m(b0) and G3m(b1), respectively, and also on all IgG1 and IgG2 (Gaardner and Natvig, 1972). The IgG4 class shows no regular genetic markers. One antigen, however, called "4a" is present on some IgG4, whereas "4b" is present on all 4a-negative IgG4 (Kunkel *et al.*, 1970). No IgG4 with both was found, but 4a is present on all IgG1 and IgG3, and 4b is present on all IgG2. Since normal sera are positive for these "nonmarkers", they do not serve as genetic markers in the ordinary sense (hence, the name "nonmarkers"), but they have been important as markers in isolated subclasses of IgG. For example, Michaelson and Natvig (1971) devised a procedure for separating IgG1 and IgG3 Fc fragments from normal serum, and could use non-a and non-g as markers. Abel (1972) has demonstrated the amino acid differences in IgG4 accounting for 4a and 4b. The IgG 4a protein has a Leu residue at position 309 which the IgG 4b protein lacks.

44.2.4 Gm genetics

No other marker system, with the exception, perhaps, of HLA, shows such a high degree of variability in different racial and ethnic groups. The formal genetics of the Gm system is complicated because of the structures of the genes. Each subclass of IgG has its own cistron in the genome, and these are very closely linked to one another. Some of the marker and nonmarker genes are truly allelic, probably reflecting base changes at the same position of the structural gene. Natvig and Kunkel (1973) call these pairs "homoalleles". Examples are Glm(f)–Glm(z) [Glm(3)–Glm(17)], Glm(a)–Glm(non-a) [Glm(1)–nGlm(1)] on IgGl, Glm(g)–Glm(non-g) [Glm(21)–nGlm(21)] on IgG3, and G3m(b0)–nG3m(b0) and G3m(b1)–nG3m(b1) on IgG3. Markers in different positions within allelic genes of the same cistron are called "heteroalleles" by contrast. Because of the close linkage, they may be used as "allelic" markers in populations, though. The formal genetics of Gm is discussed by Grubb (1970), van Loghem (1971), Natvig and Kunkel (1973) and Stedman and Wainwright (1979).

As noted above, the W.H.O. Committee recommended nomenclature for the presence or absence of individual Gm factors. Glm(1) indicates that the antigen is present; and Glm(−1) indicates that it is absent. If a series of allotypic markers is expressed, the designation is written in subclass order: e.g. Glm(1,2,3)G2m(23)G3m(5,13,14). A haplotype is also expressed in subclass order, e.g. $Gm^{1,2,3;23;5,13,14}$. If G2m(23) were absent, the haplotype would be $Gm^{1,2,3;-23;5,13,14}$, or $Gm^{1,2,3;-;5,13,14}$. Phenotypes are written in subclass and haplotype order, e.g. Gm(1,2,17,3;23), where the Glm subclass haplotypes are $Gm^{1,2,17}$ and Gm^{3}, and G3m antigens were not tested. Partial phenotypes may be written which indicate only the presence or absence of antigens tested, e.g. Gm(1,2,3;-14). Stedman and Wainwright (1979) cover this material, and the other aspects of Gm in a brief but clear and understandable way. Genotypes may be written as the haplotypes, separated by a slash, e.g. $Gm^{1,17;-;21}/Gm^{3;23;5}$.

As mentioned above, various haplotypes tend to be characteristic of certain racial or ethnic groups, and some factors are very seldom present in particular population groups. One of the difficulties in population work is the rarity of some of the testing reagents, with the result that a population may be tested only for certain allotypes for which antisera are available to the investigators. Studies using antisera for many specificities are rare. Stedman and Wainwright (1979) summarized the factors and haplotypes that are relatively common in various groups: In Caucasians, $Gm^{1,17;21}$; $Gm^{1,2,17;21}$; $Gm^{3;5,10,11,13,14}$; and $Gm^{3;23;5,10,11,13,14}$; In Negroes, $Gm^{1,17;5,6,11,24}$; $Gm^{1,17;5,6,10,11,14}$; $Gm^{1,17;5,10,11,14}$; $Gm^{1,17;5,10,11,13,14}$; and $Gm^{1,17;10,11,13,15}$; In Mongoloids, $Gm^{1,17;21}$; $Gm^{1,2,17;21}$; $Gm^{1,3;23;5,10,11,13,14}$; and $Gm^{1,17;10,11,13,15,16}$. Further information may be found in Johnson *et al.* (1977).

A number of years ago, when Gm population studies were first being carried out, and the extensive biochemical data from studies of myeloma proteins was not available, there were several ways of looking at the genetics. Giblett (1969) discussed this point. Steinberg, for example, entertained at least for some time a conception of Gm genetics that was analogous to Wiener's notion of Rh genetics (see section 22.6.1) (e.g. Steinberg, 1965). Such a model postulates multiple alleles at a single locus, particular alleles being able to code for proteins that contain one or several antigenic determinants. The alternative way of looking at it imagines the series of closely linked genes in the various subclass cistrons, as outlined in the first paragraph of this section.

Extensive studies of myeloma proteins of the various subclasses, and fragments obtained from them, have resulted in the assignment of many of the markers to specific regions of the IgG chains—and thus, the genes determining them to specific regions of the cistrons (see, e.g. Turner, 1976). In some cases, amino acid sequence studies have been carried out on the chains, revealing the chemical basis for the antigenic differences. Assignment of the antigens to specific regions of the chains is discussed by Natvig and Kunkel (1973), and well summarized by Stedman and Wainwright (1979). The information is summarized in Figure 44.1. Because of the close linkage of the IgG subclass genes, different genetic events can take place because of unequal homologous crossovers. Examples are deletions, hybridizations and duplications. Families have been studied in which apparent deletions are segregating, and Natvig and Kunkel (1973) thought that this was a better explanation than silent Gm alleles. Lefranc *et al.* (1976) reported on a deletion chromosome affecting IgG3. Kunkel *et al.* (1969) have studied an example of a hybrid IgG chain, which is analogous to the "Lepore" hemoglobins (section 38.2.3.5). There is also evidence for duplication of the IgG1 genes on one chromosome (Natvig *et al.*, 1971a; Natvig and Kunkel, 1973). The arrangement of structural genes on the chromosome is thought to be in the order IgG4–IgG2–IgG3–IgG1.

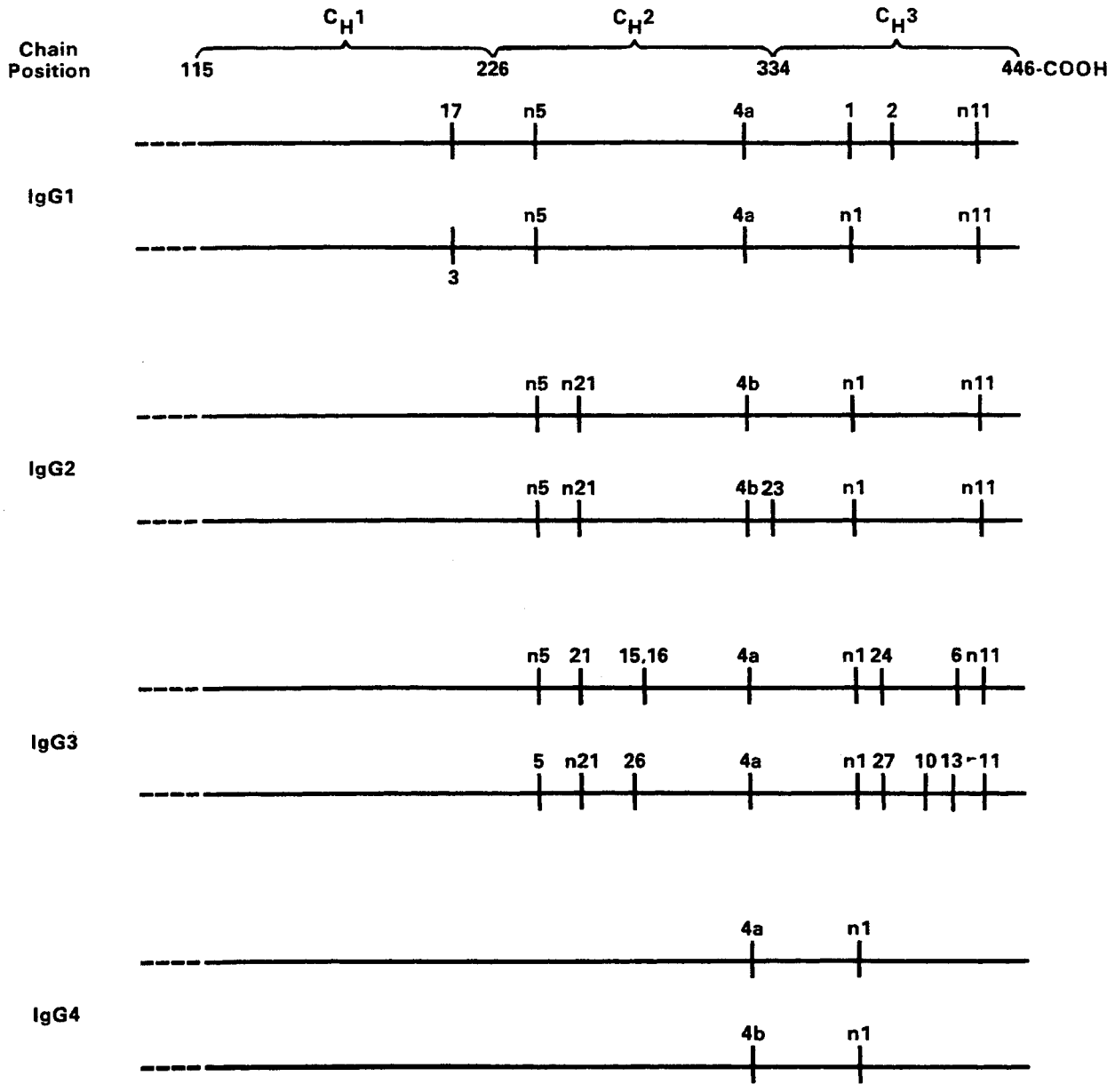


Figure 44.1 Distribution of the Genetic Markers on the IgG Subclasses
CH1 through CH3 refer to the constant homology regions.
The "nonmarkers" are denoted n1, n5, etc.

44.3 Light Chain Markers—The Km System

Ropartz *et al.* (1961a and 1961b) found an inheritable property of serum which was detected in the same way as Gm antigens, but was independent. They called it "InV", and later "Inv(a)". An additional Inv factor, called "Inv(1)" was described by Ropartz *et al.* (1962). The "(1)" in "Inv(1)" was apparently a lower case "L", but this cannot be distinguished from a number one ("1") character in printing or typing. Steinberg *et al.* (1962) described another factor called Inv(b), and it behaved as though it were determined by a gene allelic to the Inv(a) gene. Terry *et al.* (1965) showed that the Inv factors were located on the κ light chains of immunoglobulins, but not on the λ chains. In the interests of consistency, the W.H.O. Nomenclature Committee recommended that the system name be changed to "Km". Thus, as shown in Table 44.1, Inv(1) (lower case L) or Inv(1) (number one) became Km(1); Inv(a) or Inv(2) became Km(2); and Inv(b) or Inv(3) became Km(3).

The antigens of the system are inherited by way of the codominant alleles Km^1, $Km^{1,2}$ and Km^3 (Grubb, 1970). A number of populations have been tested with anti-Km(1), but the other antisera are apparently quite rare. The $Km^{1,2}$ allele is far more common than is Km^1. In Caucasians, almost all Km(1) are Km(1,2) if anti-Km(2) serum is used, and the figure is only slightly less for other populations. Similarly, over 90% of Caucasians and over 80% of Negroes who are Km(1) are Km(1,3) if tested with anti-Km(3). Km(-1,-2,-3) people are known, and the phenotype implies the existence of a Km^- gene, an inhibitor of Km expression, or of another allele to which an antiserum has not yet been found.

The amino acid substitutions in the κ chains associated with the Km allotypes have been worked out by Milstein *et al.* (1974). The residues which vary are 153 and 191. Km(1,2,-3) has 153Ala, 191Leu, Km(-1,-2,3) has 153Ala, 191Val, and Km(1,-2,-3) has 153Val, 191Leu. Additional information on Km may be found in Grubb (1970), Johnson *et al.* (1977) and Stedman and Wainwright (1979).

44.4 Other Immunoglobulin Markers

The immunoglobulin marker called "Isf(1)" (Ropartz *et al.*, 1966) received its designation because the antiserum defining it came from healthy donors from San Francisco. It is apparently independent of Gm and Km, and resides on IgG1. It is clearly and simply inherited. Ropartz *et al.* (1968) found that its expression was age-dependent in Caucasians but not in Negroes.

The first genetic marker on a heavy chain from a non-IgG immunoglobulin was independently found in IgA by Vyas and Fudenberg (1969) and by Kunkel *et al.* (1969). Like IgG, IgA molecules may be subclassified (into isotypes) according to antigenic determinants on their heavy (α) chains. The genetic marker is associated only with IgA2 molecules, and was first called Am(1) by Vyas and Fudenberg (1969) and Am_2 by Kunkel *et al.* (1969). It is now called A2m(1) to be consistent with the Gm nomenclature. A2m(1) occurred in most Whites, roughly half the Blacks tested, and in 73% of Japanese and 56% of Chinese. The numbers of people tested were small. Soon afterward, a second marker was found by van Loghem *et al.* (1973), which they called "$A_2m(2)$", and it behaved as if its gene were allelic to that for A2m(1). There are, thus, three A2m types: (1,-2), (1,2) and (-1,2). The frequency of the allele for A2m(1) is high in Caucasians, and much lower in Black and Oriental populations. Van Loghem (1974) has discussed the properties and inheritance of the A2m system. The complete amino acid sequence of an $\alpha 2$ heavy chain from immunoglobulin molecules of the A2m(2) allotype has recently been determined (Toraño and Putnam, 1978).

All the IgG markers described above reside on the constant homology regions of the heavy chains. A marker was recently described on the variable regions of the heavy chains of IgG, IgM and IgA (Wang *et al.*, 1978). It was called "Hv(1)".

44.5 Determination of Immunoglobulin Types

There are essentially only two ways of determining immunoglobulin types. The first is serological, and is probably the most widely used. The second is immunoprecipitation using precipitating antibodies raised in animals. Precipitating anti-Gm antibodies can be prepared to some antigens in rabbits, and in primates such as the rhesus monkey or the baboon (Hess and Bütler, 1962; Alepa and Steinberg, 1964; Litwin and Kunkel, 1966c; Yount *et al.*, 1967; Natvig *et al.*, 1968; Van Loghem and Grobbelaar, 1971).

The serological method is based on antibody neutralization (inhibition test). The presence of an antigen in a sample is indicated by its ability to prevent agglutination of red cells coated with specific IgG molecules which have the antigen after incubation with the antiserum. The serological test, therefore, requires two reagents: the anti-Gm reagent (often called the "agglutinator"), and an IgG incomplete antibody, containing the Gm factor, which will sensitize the red cells without agglutinating them (often called the "antibody coat" or simply the "coat"). Anti-D is customarily used as a coat, in part because of its availability, and in part because of its sufficiently higher titer. There can be variability in the behavior of both reagents of their pair, even though they may appear to have the same specificity. The differences between Ragg and SNagg agglutinators have been noted. The latter are preferable if available. There can be variability in the antibody coat as well, and these reagents must be carefully selected and standardized. Giblett (1969) noted that Rh+ cells with as many D sites as possible should be employed (section 22.7.1). Some Gm specialists suggest that R_1R_1 cells are preferable to R_2R_2 cells. The sensitized cells should be strongly Coombs positive. Giblett (1969) discussed both tube and tile techniques. Borel *et al.* (1967) described a microprocedure on microtiter plates. These reagents are often very precious, and microtechnique is preferable wherever possible. A special technique was described

in section 44.2.1 above for artificially coating red cells with appropriate myeloma proteins to detect a specificity for which a proper anti-D coat could not be found. A clearly presented and very useful methods monograph has recently been published by Kipps (1979).

44.6 Medicolegal Applications

44.6.1 Disputed parentage

The Gm system has been employed in disputed parentage cases in specialized laboratories for quite some time. This application is discussed in detail by Planques *et al.* (1961), Harboe and Lundevall (1961), Prokop and Hunger (1961), Prokop and Bundschuh (1963), Ellis *et al.* (1973), van Loghem and Nijenhuis (1979) and Sebring *et al.* (1979), among others. The value of Gm typing in parentage cases depends on the number of specificities which can be tested, and this varies to some extent from one laboratory to another. In this country, the use of Gm in paternity testing seems to be more common in the AABB Reference Laboratories (Polesky and Krause, 1977). In the older papers, fewer specificities were employed for the testing than in the more recent ones.

There is general agreement that the system is very useful, and improves the overall exclusion percentages provided that the typing is carried out skillfully and that all the intricacies of Gm and Km genetics are appreciated. The article by Sebring *et al.* (1979) provides excellent coverage of the subject. They presented results on 925 cases in which Gm typing was carried out using anti-Glm(1), (2), (3), (17), G3m(5), (10), (6), (24), (21), (15), (16) and Km(1). G2m(23) typing was employed to sort out a case involving an uncommon haplotype. They said that the exclusion probability was about 24% for Caucasians and 17% for Negroes in this country, using anti-Glm(1), (2), (3) and anti-G3m(b1). The figure would increase to about 33% for Black Americans if Glm(17), G3m(21), (10), (6), (24) and (15) were added.

44.6.2 Gm and Km typing in bloodstains

Efforts to group Gm antigens in bloodstains began about 20 years ago, and it is well established that reliable Gm typing is possible with bloodstains. In practice, it appears that Gm typing is limited to specialized laboratories that have developed experience with the system. Km antigens can likewise be determined in bloodstains.

Among the most extensive earlier studies on Gm stain typing are those of the group in Toulouse. Background for the serum group systems was presented by Ruffie (1961). In 1961, Planques *et al.* presented their studies on the determination of Glm(1), Glm(2) and G3m(5) in bloodstains. Stains were deposited on a number of substrata, both absorbent and nonabsorbent, and both Ragg and SNagg reagents were tested. Dried blood on nonabsorbent substrata were easier to work with. Nonspecific absorption of the anti-Gm was not observed, but they said that the SNagg antisera gave more consistently complete inhibitions with stains containing the corresponding Gm antigen than did Raggs. There was no doubt that all the antigens tested could be grouped specifically and reliably in stains. In some cases, stains that had been stored for years could be typed, attesting to the stability of the Gm antigens. Further studies on the typing of Glm(1), Glm(2) and G3m(5) were carried out by Ducos *et al.* (1962 and 1963a). In Germany, similar studies were done by Fünfhausen and Sagan (1961), Fünfhausen *et al.* (1962) and Sagan and Fünfhausen (1965). They studied Glm(1) and Glm(2) determination in bloodstains on a variety of substrata. Even stains exposed to 100° conditions for an hour (7 days old stains) could still be typed. In 1962, Prokop *et al.* devised a rapid microprocedure for Gm typing, which was applicable to bloodstain typing. Ducos *et al.* (1963b) briefly reiterated the value of Gm typing. Kobiela (1963) confirmed that Glm(1) was reliably determinable. Nielsen and Henningsen (1962) also confirmed that Gm factors could be determined in bloodstains, as did Lenoir and Muller (1966) and Turowska (1969). The factors were not detectable in older stains kept at higher humidity conditions, but the stains kept dry seemed to retain activity well. In 1964, Brocteur and Moureau reported that they had been able to sort out a car accident case, involving a determination who had been driving one of the cars, based in part on Gm typing of a bloodstain in the car, and comparison of the result with the Gm types of the occupants. A lengthy and extensive study of Gm stain grouping was carried out by Görtz in 1969. The conditions of the inhition test were studied in detail, and he tested more than 950 bloodstains. Under favorable conditions, Glm(1) and Glm(2) could be typed using 0.1 mg of stain material, somewhat more being required for G3m(5). Stains which were still comparatively soluble were more readily groupable than those which had become insoluble upon aging. Like other investigators, he emphasized the careful selection and evaluation of reagents for the tests. The studies extended to Glm(3), G3m(6) and G3m(10) as well, indicating that all could be reliably determined. Görtz said that negative inhibition results had to be interpreted with great caution in older, insoluble stains, since these might fail to inhibit the agglutinator even though the corresponding factor was present. A full paper on Glm(3) appeared in 1969, emphasizing that information about the racial origin of stains could be obtained by combining Glm(3) and G3m(5) typing (Ducos *et al.*, 1969). The Gm(-3,5) type was characteristic primarily of Black people (cf. above in section 44.2.4). Stains as old as 15 years could be typed. In 1971, Blanc and Görtz reported on the typing of Gm(Bet) [G3m(13)] in bloodstains. In this paper, they showed that typing results in sera and the corresponding bloodstains were concordant for Glm(1), (2), (3), G3m(5), (6), (10), (13) and for Km(1) and (2). Blanc *et al.* (1971) discussed the value of Gm phenotyping in bloodstains in terms of determining racial origin. Certain haplotypes and phenotypes occur in very much higher frequency in certain populations than others, as noted in 44.2.4, and careful selection of the factors to be tested can be informative. They also noted that only the positive identification of an antigen should be used in the interpretation. Görtz *et al.* (1970) discussed the Gm typing technique and possible

sources of error. The agglutinator should be SNagg, and have a titer of 1:16 to 1:32. They seemed to think, too, that the coat reagents should be prepared in specialized laboratories. Most of the same types of problems encountered with any kind of stain grouping are encountered in Gm typing as well.

Km antigens can be determined in bloodstains. There are a number of reports on Km(1) (Merli and Rouchi, 1967; Blanc and Görtz, 1971; Blanc *et al.*, 1973; Khalap *et al.*, 1976), and on Km(2) (Blanc and Görtz, 1971; Blanc *et al.*, 1973).

Khalap *et al.* (1976) described a procedure for the typing of Glm(1), Glm(2) and Km(1) in stains. This procedure appears in the MPFSL (1978) manual as well. They noted that woolen substrata can give "cloth reactions," i.e., nonspecific inhibition of the agglutinator. Khalap and Divall (1979) reported on the typing of G3m(5) in bloodstains. The antigen was reliably determinable, and they noted that the addition of G3m(5) to their routine testing protocol had additional value in the interpretation of negative results with Glm(1) and Glm(2). A substantial number of the samples tested in London (from British Caucasians) were Gm(−1,−2;5). If only Glm(1) and (2) were included in a test on a stain from such a blood, the negative results would probably be uninterpretable. However, if G3m(5) is found, the likelihood is much stronger than the Gm(−1,−2) result actually represents the type and is not a reflection of deterioration of the antigens. They noted that G3m(10) could be used in a similar way. This matter is clearly discussed by Shaler (1982) as well, using casework illustrations. A negative result for a factor can be interpreted as such if a positive result is obtained in the same sample for another factor residing on the same IgG subclass. A very detailed discussion of Gm and Km typing procedures in forensic samples was given by Kipps (1979). Davie (1979) described a procedure for Gm and Km typing on microtiter plates.

The immunoglobulin markers may represent the only genetic marker system used in forensic serology which by their nature allow some control over the interpretation of negative results. In general, failure to detect an antigen in a bloodstain cannot normally be taken to mean that it was never present. In the case of some Gm antigens, however, the finding of one specificity can be used as a positive control for the presence of the immunoglobulins in the stain, as Khalap and Divall (1979) have noted. In this way, it may be possible to interpret negative reactions with more confidence. Such positive marker control antigens are even better if they reside on the same subclass of IgG molecules. One wonders if the so-called nonmarkers, or antisera defining particular subclasses (isotypic), could not be put to good use as indicators of the presence of the IgG molecules containing the Gm factors being sought.

Perhaps the most appealing property of the Gm and Km system antigens for bloodstain work is their extraordinary stability. No other genetic marker known, except for ABO, shows anything even approaching the stability of the Gm antigens in dried blood. Various Gm and Km factors have been typed in bloodstains many years old (Görtz, 1969; Ducos *et al.*, 1969; Blanc *et al.*, 1973). Budyakov (1967) detected Glm(1) in 5 year old stains regardless of exposure to often adverse environmental conditions. Similarly, Turowska (1969) found that Glm(1) and Glm(2) could be typed in 5 year old stains. Hoste *et al.* (1978) recently detected the Glm(1), G3m(10) and Km(1) types in 33 year old bloodstains.

The greatest difficulty with the Gm system is the availability of reagents. SNagg reagents and appropriate anti-D (or even other) coats for many of the specificities are not very common. Antisera can be developed in animals, but unless there is more commercial interest in such reagents, they will probably continue to be rather hard to obtain for some time. Since the reagents are ordinarily precious, it is useful to develop systematic approaches to Gm typing, taking into consideration the phenotypic distributions in the population of interest, so as to be able to obtain the maximal discrimination using the fewest reagents. Such a flowsheet for the English population is given by Stedman and Wainwright (1979).

44.6.3 Gm and Km typing in body fluids and tissues

Any tissue or fluid that contains immunoglobulins of the appropriate classes will contain the associated genetic markers, but not necessarily in concentrations adequate for typing, particularly in stains. Nielsen and Henningsen (1963) could not detect Gm factors in 23 seminal specimens. Klose and Schraven (1962) had detected Glm(1) in some specimens from secretor patients, but reported to Nielsen and Henningsen (1963) that they could not always detect it in semen from healthy donors. Krämer (1963) found Glm(1) in 9 of 30 seminal samples. Davie and Kipps (1976) showed that seminal Km(1) types match the type in serum from the same donor (31 samples). Special techniques were needed for grouping liquid semen (or saliva) samples because of their ability to cause nonspecific aggregation of the test cells. A freeze-thaw step, followed by centrifugation, prevented this effect in most cases. Many typing reagents perfectly suitable for serum typing were not very satisfactory for semen and saliva, because of the low immunoglobulin concentrations in these fluids. Jorch and Oepen (1977) reported that a number of Gm factors and Km(1) could be detected in semen if the quantity were sufficient, but that contamination by other body fluids which contained immunoglobulins was a danger in medicolegal samples. It was considered more likely that markers on IgG1 would be detectable than those on IgG3 in semen. Both nasal and vaginal mucus were said to be possible sources of problems if they were found in mixtures with semen. Blake and Sensabaugh (1978) noted that there is about 50 times more IgG in blood than in seminal plasma, and that one would expect to require at least the equivalent of 0.1 mℓ of semen to detect an IgG marker. Davie and Kipps (1976) reported some limited success with seminal stains, but said that more sensitive techniques would be needed.

In saliva, immunoglobulin levels are about 1000 times less

than in blood (Waissbluth and Langman, 1971). Krämer (1963) did not detect Glm(1) in normal saliva. Davie and Kipps (1976) found that Km(1) types correlated in paired saliva and semen samples, but the saliva had to be treated by freezing, thawing and centrifugation to remove its red cell aggregating property. Only selected examples of anti-Km(1) were found to be suitable for this typing. Some success was reported with saliva stains, but increased sensitivity was needed in the test because of the relatively low immunoglobulin levels. Km(1) was selected because it is not restricted to IgG, and might be expected to be present in somewhat higher concentratons than Gm factors. Jorch and Oepen (1977) found that Km(1) could be detected in saliva and saliva stains, but they had no success with Gm factors.

Gm factors appear to be detectable in urine only if it contains a pathologically high protein concentration (Nielsen *et al.*, 1963). Sweat does not have levels of immunoglobulins sufficient to permit Gm or Km antigen detection, but nasal and vaginal mucus do have (Jorch and Oepen, 1977). Caution must therefore be exercised if mixtures are suspected, and typing is to be attempted. Human inner ear fluid can be typed for Glm(1) and Km(1) (Turowska and Trela, 1977). Tausch *et al.* (1977) studied the possibility of detecting Glm(1), (2), (3), G3m(10) and Km(1) in decaying blood and organ materials. Serum remained typable the longest as a rule. Organ exudates were typable for varying lengths of time, from 1 to 8 weeks. Henke and Bauer (1980) recently reported that a number of Gm factors and Km(1) can be detected in the pulp material of human teeth.

44.7 Distribution of Gm and Km Phenotypes in Populations

There are a number of studies on various U.S. populations, but they differ in the number of antigens determined. An older study by Blumberg *et al.* (1964a) tested Glm(1), (2), G3m(5), (6), Km(2) and Km(3) in both White and Black populations of southeastern Georgia. The best overall reference is probably Johnson *et al.* (1977).

SECTION 45. OTHER SERUM PROTEIN GENETIC MARKERS

45.1 Serum Lipoproteins—The Ag, Lp and Ld Systems

45.1.1 Introduction

The lipoproteins are a heterogeneous and complex group of serum proteins. They are macromolecular complexes having reproducible lipid-protein ratios and stability in aqueous solution. There are several ways of classifying lipoproteins, each based on a different separation criterion. The nomenclature is imprecise and sometimes ambiguous, and there is no universal agreement on it as yet. In part, this situation is due to the complexity of the systems, and in part it is due to the rapid expansion of knowledge in the field. An *apolipoprotein* is a homogeneous protein, containing no detectable noncovalently bound lipid. A *lipoprotein* is an apolipoprotein-lipid complex held together by noncovalent forces. The lipid molecules are arranged such that there are no clearly defined surface areas where polar groups shield intramolecular hydrophobic regions from solvent. A *lipoprotein particle* is an apolipoprotein-lipid complex, again held together by noncovalent bonds, except that here there are extensive regions where polar groups of lipids form a surface to shield intramolecular hydrophobic regions from solvent. These useful definitions were given by Osborne and Brewer (1977) in their extensive review of plasma lipoproteins.

Lipoproteins have been classified on the basis of (1) electrophoretic mobility; (2) hydrated density; and (3) apolipoprotein composition. According to electrophoretic migration criteria, lipoproteins may be defined as those at the origin (nonmigrating) and those migrating with pre-beta (α_2), β and α (or α_1) mobilities on paper or agarose. The migration may be a little different on starch blocks (Scanu *et al.*, 1975). Hydrated density is the most widely employed classification criterion. The relatively low density of these proteins, compared with other serum proteins, is an advantage in separating them. They were originally classified as: chylomicrons ($\rho < 1.006$), very low density lipoproteins (VLDL; $\rho < 1.006$), low density lipoproteins (LDL; $\rho = 1.006$–1.063), and high density lipoproteins (HDL; $\rho = 1.063$–1.21). Refinements of technique have resulted in the division of LDL into two classes and of HDL into three. So-called very high density lipoproteins (VHDL; $\rho = 1.21$–1.25) have also been recognized. Apolipoprotein composition studies of the varous density fractions has led to a classification of lipoproteins into families, based on the apolipoprotein composition. The families are called Lp A through Lp E, and each contains characteristic proteins or peptides, some of which have been better characterized than others. The specific peptide or protein constituents of Lp A are called A-I and A-II; those of Lp C are C-I, C-II, etc. This material has been thoroughly reviewed by Scanu *et al.* (1975) and by Osborne and Brewer (1977).

The polymorphic serum lipoprotein antigens that are discussed in the remainder of section 45.1 are associated with the low density lipoproteins (LDL).

45.1.2 The Ag system

The first antigen of the Ag system was observed by Allison and Blumberg in 1961. There had been previous observations in animals, and even in human beings, of the formation of precipitating antibodies by one individual against the serum proteins of another in response to transfusion. No thorough or systematic studies had ever been done on these "isoprecipitins", however. The formation of such antibodies indicates serological variations in the serum proteins within the same species. Allison and Blumberg (1961) described a precipitating antibody in the serum of a patient at N.I.H. (Mr. C.deB.) who had received many transfusions. The antibody reacted with some but not all examples of human sera from other people. The antigen in human serum that was reacting with C.deB. serum was called "Ag" (which just stood for "antigen"). The Ag factor was inherited in a simple Mendelian way, and Blumberg *et al.* (1962a) said that the gene determining the antigen should be called Ag^A, and its allele Ag. Ag^AAg^A and Ag^AAg people would thus be Ag(a+), while $AgAg$ ones would be Ag(a−). Ag(a+) was shown to be associated with the low density (β) lipoproteins of serum (Blumberg *et al.*, 1962b). In 1963, Blumberg and Riddell described another precipitating antibody in a patient's serum (the 'New York' serum) which defined an apparently different Ag factor, and this was called Ag(b). It was thought to be conditioned by another gene, Ag^B, and Blumberg (1963) suggested that this gene was allelic to Ag^A, at least in some populations. Blumberg and his collaborators continued to screen the sera of multiply transfused patients for precipitating antibodies, and, by 1964, they said that the antibodies all taken together defined from 5 to 7 separate antigenic specificities (Blumberg *et al.*, 1964b). Information about the various antisera, and about the frequencies of their corresponding antigens, and their relationships and inheritance, was not very detailed, however. In addition, it was apparent by then that some of the antisera were polyspecific. The very early studies with Allison were discussed by Blumberg (1977) in his Nobel address. The study of antibodies in polytransfused patient sera led Blumberg and his collaborators off in a different direction once the "Australia" antigen had been discovered (section 50.2.3), and these studies culminated in the discovery of the infectious agent responsible for hepatitis B. Blumberg shared the Nobel Prize for Physiology or Medicine in 1976 for the hepatitis work.

Hirschfeld and Blombäck (1964) described another anti-Ag serum (the "L.L." serum) which reacted with about 40% of Swedish sera, and had a peculiar relationship to C.deB. serum. All C.deB.-negative samples were also L.L.-negative, but C.deB.-positive sera could be either L.L.-positive or L.L.-negative. The relationship was similar to that existing between Gm(a) and Gm(x) (see in section 44), and the specificity in the L.L. serum was thus named Ag(x). C.deB. serum was found to contain not one, but three different Ag antibody specificities. One of these, anti-Ag(x), it shared with L.L. serum. The other two were named anti-Ag(a_1) and anti-Ag(z) (Hirschfeld *et al.*, 1964). These findings tended to simplify somewhat the conception of the Ag system (Hirschfeld, 1968b). Some of the antisera shared antibody specificities, and were polyspecific, and some of the earlier complexities could be understood in these terms, rather than in terms of relationships between the different antigens. The original so-called "Ag(a)". and its gene "Ag^A", disappeared in the revised interpretation.

A number of different isoprecipitin sera have been described, and sorting out the different specificities and their immunological and genetic relationships has not always been an easy task. Until around 1964, there were really only three fairly well characterized antisera, the C.deB., the New York, and the L.L. ones. C.deB. was an anti-Ag(a_1,x,z). The L.L. serum was considered to be anti-Ag(x), and possibly the only monospecific reagent of the three, since the New York serum was suspected of being more than just anti-Ag(b) (Blumberg *et al.*, 1964b). In 1966, Geserick *et al.* found an "anti-Ag(b)" antibody in a patient in Berlin who had been transfused over 200 times. It reacted with about 93% of German sera. Hirschfeld *et al.* (1966) described another serum ("B.N.") which appeared to define a new specificity called Ag(t). It seemed that B.N. serum was a monospecific anti-Ag(t). Then, in 1967, Hirschfeld and Bundschuh compared the Berlin example of "anti-Ab(b)" (the "New York" serum was no longer available) and the "B.N." [anti-Ag(t)] serum, and said that they were identical. Another serum, called "C.P.", was described by Hirschfeld *et al.* (1967). It reacted with an antigen in about 90% of Italian and Swedish sera, and was thought to be defining a new antigen, Ag(y), whose determining gene, Ag^y, appeared to be allelic to Ag^x. Two other sera sent in by other workers for evaluation appeared to be anti-Ag(y) as well. Data in a number of populations were consistent with the assumption that Ag^x and Ag^y were allelic (Hirschfeld and Okochi, 1967), and examples of anti-Ag(x) and anti-Ag(y) were found in the Japanese population (Okochi, 1967). Bütler *et al.* (1967a) examined eight serums with anti-Ag activity. Six had anti-Ag(x), and in two, an additional antibody defining a new specificity called Ag(r) was found. One serum appeared to have a monospecific anti-Ag(r), and another, a monospecific anti-Ag(y) as well. In 1966, Bütler and Brunner described a passive hemagglutination technique for typing Ag specificities. It was sensitive, and offered a number of advantages compared with the double diffusion technique which had been employed exclusively up to that time. Red cells were "coated" with LDL of known Ag specificity by a diazotization procedure. Corresponding anti-Ag sera would then give agglutination of the cells. This kind of "coating" technique was introduced by Coombs *et al.* (1952) and discussed in section 16.3. It was further discussed in section 44.2.1 in connection with the determination of G2m(23) [G2m(n)] for which a suitable anti-D "coat" was never available. Bütler *et al.* (1967b) described an anti-Ag activity which was not precipitating, and could be detected only by the passive hemagglutination technique. This antibody defined an apparently new Ag specificity called Ag(c). Further studies on Ag(c) and another new specificity, Ag(e), were reported by Bütler and Brunner (1969). In 1968, Contu found a serum ("R.M.") which appeared to define another Ag antigen, which he called Ag(m). He found that all "L.L. serum"-positive sera were Ag(m+) while all "L.L. serum"-negative ones were Ag(m−). Although "L.L." serum was regarded as an anti-Ag(x), Contu thought that it was probably anti-Ag(x,m). Bütler *et al.* (1970a) described polyspecific antisera which contained, in addition to anti-Ag(a_1) and anti-Ag(x), a new anti-Ag(g). Bütler *et al.* (1970b) described two sera containing antibodies to yet another specificity, Ag(d). Boman (1971) described an antiserum whose activity was detected by passive hemagglutination technique. This was not an anti-Ag(x), but its relationship to the other factors is not clear.

The complexity of the Ag system, and the difficulty in understanding the relationships between the antigens, can be accounted for in several ways. Antibodies are apparently found only in the sera of people who have received many transfusions. Many of the antibody-containing sera have several antibodies in them. In addition, anti-Ag sera are comparatively rare. Geserick and Dufková (1967) found an anti-Ag(b) [anti-Ag(t)] in the serum of a patient who had received 205 transfusions. After 12 additional transfusions, an anti-Ag(x) developed in the individuals's serum. Vierucci *et al.* (1966) reported screening 80 sera from multiply transfused patients, and finding anti-Ag activity in 6 of them. Hirschfeld (1968b) said that about 60 reagents had been described by the time that review was written. There are technical problems and variations associated with Ag typing as well. It is difficult to "standardize" the test procedure because of variations in the strengths of antisera, and perhaps because of differences in the reactivity of the same lipoprotein antigen in different sera. Hirschfeld (1968b) conducted extensive studies on 28 different isoprecipitin sera with a panel of 462 sera from unrelated individuals. Particular antisera do not always give the same results with the same panel serum in repeated tests over the course of time, although the great majority of repeated determinations were concordant. The "L.L." serum, for example, tested with 108 panel sera in 536 determinations gave deviant results in 15 instances. Some series of immunodiffusion plates give better results than others, and nonspecific precipitin zones, formed around the antigen wells, can sometimes cause problems of interpretation. Incubation time is another variable that is difficult to optimize because it is dependent on which

reagents are used, and the amounts of each, the distance between the wells, and the temperature. Polyspecific reagents can contain antibodies with different optimal incubation times. There can be differences in the reactivity of reagents at different times during storage as well. Sera kept at −20° sometimes showed negative results after having reacted with the same antiserum when they were fresh. In a few other cases, sera previously determined to be negative with a particular antiserum reacted with it after being stored frozen for 2 years. These technical factors obviously have an effect on the results obtained with particular reagents, and the subsequent conclusions that are drawn about the Ag system.

Much of the evidence (Morganti *et al.*, 1970; Bütler *et al.*, 1971; Hirschfeld, 1971) tends to support a genetic model for the Ag system that postulates four closely linked loci controlling a total of eight antigens. $Ag^{x,y}$ can designate the locus at which Ag^x or Ag^y alleles may be found. The other three loci are $Ag^{a_1,d}$, $Ag^{c,g}$ and $Ag^{t,z}$. This model permits 16 different chromosomal arrangements, which can theoretically give rise to 136 genotypes and 81 phenotypes. Not all of them have been found. In addition, Ag^z is never present in the absence of Ag^{a_1} (Hirschfeld, 1971). The four closely linked loci model is not the only possible one. A single locus for the Ag system with multiple alleles is conceivable, if each allele produces a product with four different antigenic determinants, or factors. These two models are closely analogous to the Fisher-Race and Wiener conceptions, respectively, of Rh (section 22.6.1). Hirschfeld (1971) has noted that, in either case, antibodies are regarded as being monospecific (simple) while each Ag chromosome produces four different antigens which are inherited as genetically nonsegregating units ("complex antigens"). He therefore calls these models "simple-complex". One can imagine other kinds of models in which the chromosome produces only one antigen with a single determinant, and where antibodies are complex, i.e., they can react with the product of more than one Ag chromosome. If both antigens and antibodies are allowed to be complex, even more involved models can be constructed (Hirschfeld, 1971). While it is convenient to think of Ag system genetics according to a model having four closely linked loci, there are undeniable problems with it. Apart from those mentioned above, it is not clear where Ag(m) fits into the picture. Bütler *et al.* (1971) said that Ag^m might be an allele of Ag^x and Ag^y. Bütler *et al.* (1974) have reported an additional specificity called Ag(h), thought to be conditioned by an allele Ag^h. This allele was said to occupy a fifth closely linked locus, and its hypothetical partner was designated $Ag^{[i]}$. Giblett (1977) brought up the interesting idea that the five Ag loci, assuming the model is correct, might have a correlation with the five classes of LDL, in a manner somewhat analogous to the relationship between the Gm antigens and the IgG subclasses (section 44).

The Ag system was reviewed by Bütler (1967a), Bütler *et al.* (1971) and Hirschfeld (1968b and 1971). Allison (1963) noted that the Ag system was not a particularly promising prospect for forensic blood identification because the antisera were so rare. For this reason, and perhaps others, the system has been used very little in medicolegal work. The Ag(x)-Ag(y) pair, which is believed to be conditioned by a pair of alleles at a single locus, has been employed as a marker in paternity cases in the United Kingdom (Bradbrook *et al.*, 1971). According to Lee (1975), the PE for a falsely accused White father would be about 14% using Ag(x,y) testing.

45.1.3 The Lp system

In 1963, Berg reported that he had detected a new, genetically controlled serum lipoprotein antigen in humans. Serum produced in rabbits by immunization with human LDL, and then absorbed with various human sera to remove the "anti-LDL" activity, retained a precipitating activity against about 35% of sera from unrelated donors. The antigen being detected was called "Lp(a)", where "Lp" stood for "lipoprotein". Reactors were designated Lp(a+), and nonreactors were Lp(a−). The gene postulated as being responsible was called Lp^a, and it behaved as a mendelian dominant. Further genetic studies (Berg and Mohr, 1963) indicated that the genetic model was probably correct. The hypothetical allele of Lp^a was designated Lp^a. In one family, however, an Lp(a−) × Lp(a−) mating had produced an Lp(a+) child. While there was no evidence of illegitimacy, it could not be ruled out either. Lp(a) was different from any of the antigens of the Ag system (Berg, 1964a). Problems could be encountered in typing Lp(a), and Berg (1964b) discussed an albumin-β-lipoprotein precipitation which he said could be a source of misinterpretation in reading immundiffusion gels. Lp(a) was not very stable to storage, or to freezing and thawing, and Berg (1964c) noted that it should be typed in fresh serum. Protocols for preparing rabbit anti-Lp(a) were given by Berg (1965a), and he also found (1965b) that the antibody the rabbits made was a 7S γ-globulin. Additional family and population studies (Berg, 1966) were consistent with the proposed genetic model. Wassenich and Walter (1968) reported on another family, however, in which an Lp(a−) × Lp(a−) mating had produced three Lp(a+) children (out of a total of ten). The *Lp* locus is apparently not closely linked to *Ag* (Berg, 1967).

In 1964, Bundschuh found that "anti-Lp(a)" produced in horses behaved differently from the rabbit reagent. Two precipitin lines were produced against Lp(a+) sera, one showing immunologic identity with Lp(a) and one showing nonidentity. Prokop and Uhlenbruck (1969) discussed these studies in more detail. The horse antiserum was thought to be detecting a different but related antigen, called Lp(x). All Lp(x+) sera were Lp(a+), but not conversely. The relationship between Lp(a) and Lp(x) is not very clear. Berg (1968) tested the horse serum as well, and confirmed the results of the German workers.

Simons *et al.* (1970) partially purified the Lp(a) lipoprotein. They said it differed from LDL and HDL in amino acid composition, but was similar to the former in lipid composition. They further said that the protein had a characteristic mobility upon disc electrophoresis, and that this

technique might be used for Lp typing. Utermann and Wiegandt (1970) found that they could not characterize Lp(a+) sera by gel electrophoresis. Rittner (1971) reported electrophoretic variation in the lipoproteins using disc electrophoresis. This appeared to be genetically controlled, but its relationship to Lp is not very clear. Seegers *et al.* (1965) also described an inherited electrophoretic variation in the β-lipoprotein pattern, and this too may be related in some way to Lp.

In 1970, Harvie and Schultz found that Lp(a−) sera contained measurable quantitites of Lp(a) when concentrated. They regard the Lp polymorphism, therefore, as being a quantitative genetic trait. Utermann and Wiegandt (1970) interpreted their electrophoretic results as being consistent with Lp(a) as a quantitative trait. Enholm *et al.* (1971) found that serum Lp(a) levels varied over a very wide range (about 30-fold), and this finding would be consistent with quantitative variation. Albers *et al.* (1974) measured Lp(a) concentrations by radial immunodiffusion, and did not detect bimodality in the concentration distribution. They concluded that the variation was consistent with the polygenic inheritance. Sing *et al.* (1974) and Schultz *et al.* (1974) carried out quantitative studies on Lp(a) in serum using a sensitive radioimmune inhibition technique. They found that all individuals had Lp(a), but that the concentrations were distributed bimodally. They imagine that Lp^a, the dominant allele, determines one distribution of Lp(a) concentrations, while the allele determines another distribution with a lower mean value. Fisher *et al.* (1975) have found that human LDL seem to exist in several different MW classes, and that the classes possessed by a given individual are under genetic control, apparently at a single locus. The relationship of this finding to the Lp system is not yet clear, but it may be related to the concept of Lp(a) as a quantitative genetic variant. The studies of Sing *et al.* (1974) and of Schultz *et al.* (1974) indicated that there is a slight overlap in the Lp(a−) concentration range and that of Lp(a+) people. In this view, the Lp(a+) and Lp(a−) phenotypes are not unambiguously related to genotype. This model would help to explain some peculiarities of the system, the most troublesome of which is the occasional observation of Lp(a+) children from Lp(a−) × Lp(a−) matings.

The Lp(a) protein has been isolated in a number of different ways, and characterized to some extent (Enholm *et al.*, 1971; Utermann and Wiegandt, 1969; Dubarry and Moullec, 1976; Desreumaux *et al.*, 1977a and 1977b). The Lp(a) lipoprotein is closely related to LDL in some respects, but is apparently not identical with them. Upon storage or detergent treatment, the Lp(a) preparation may dissociate into LDL, albumin and Lp(a) apoprotein.

The Lp system has been reviewed by Bütler (1967), Berg (1968 and 1971a) and Cooper (1978). There have been almost no reports on applications of Lp to forensic or medicolegal problems. In view of the complexities of Lp(a), many of which are not yet completely understood, it is probably not a very suitable system for forensic work. Haferland (1965) did studies on the detection of Lp(a+) in 24 hour old dried blood on filter paper. He optimized the pH and buffer concentration of the buffer used to make the immunodiffusion gels, and then tested a number of samples. The antigen was detected in 20 of 21 Lp(a+) samples, and the instability of Lp(a) was noted.

45.1.4 The Ld system

Berg (1965c) found an isoprecipitin in the serum of a multiply transfused boy which reacted with about 42% of the sera of unrelated people. The antigen was associated with LDL, and was called "Ld(a)". It was not related to the Lp system, and reacted best at lower temperatures. Its controlling allele was called Ld^a, which behaved as a Mendelian dominant in family investigations. Berg and Reinskou (1967) found that the anti-Ld(a) was an IgG antibody. Berg (1971a) said that the original serum had later been found to contain both anti-Ag(x) and anti-Ld(a), but that monospecific antisera for Ld(a) had been found. Another antiserum had also been found which appeared to react with the product of the allele of Ld^a.

45.2 Complement Components

45.2.1. Introduction

The complement system was discussed briefly in section 1.3.5. Genetic variation has been found in a number of the components of the complement system, and both quantitative and structural variants have been reported. Complement component variants are typed by electrophoresis or by isoelectrofocusing, and may be detected by protein staining, immunofixation, or by functional detection overlays, depending upon the component. A recent review of this material may be found in Hauptmann (1979), and other reviews are Cooper (1978), Hobart and Lachmann (1976) and Gitlin and Gitlin (1975). The complement component deficiency states, which are under genetic control, were reviewed by Agnello (1978).

45.2.2 C2 Component

C2 is a single chain protein of approximate MW 102,000. Genetic variation in this component was detected by Hobart and Lachmann (1976), and independently by Alper (1976). There are two or three prominent bands and several additional minor bands in the C2 patterns of most individuals. C2 patterns are usually determined by polyacrylamide gel isoelectrofocusing (PAGIF), and detected by functional techniques, using an agarose overlay containing sensitized red cells and a C2-deficient serum. Alper (1976) called the common pattern "C2–C", and it accounted for about 96% of the samples. The rarer variants are of two kinds, acidic and basic, and were designated "C2–A" and "C2–B". These were accounted for by three alleles, $C2^C$, $C2^A$ and $C2^B$. Meo *et al.* (1977) studied this polymorphism further. They used the designation "C 2–1" for the common type, and "C 2–2" designated the rare basic type. The rare acidic type would be "C 2–3". The responsible alleles are $C2^1$ (= $C2^C$), $C2^2$ (= $C2^B$) and $C2^3$ (= $C2^A$). Olaisen *et al.*

(1978) confirmed these findings, and they used the designations of Meo *et al.* (1977). $C2^2$ was found to be rare in the Norwegian population as well. C2 deficiency is the most common deficiency of a classical complement component. The deficiency is inherited and quite rare. In view of the finding of structural variation at the *C2* locus, the deficiency states are now usually attributed to homozygosity for a rare silent allele $C2^\circ$ (Klemperer, 1974). The *C2* locus is linked to the *HLA* locus (Fu *et al.*, 1974; Alper, 1976; Meo *et al.*, 1977; Olaisen *et al.*, 1978).

45.2.3 C3 Component

C3 is a pivotal protein in the complement system. It occurs in relatively high concentrations in human serum, and it can be detected comparatively easily by simple staining for protein after high voltage electrophoresis. For these reasons, it is perhaps the best studied of the complement component polymorphisms.

The genetic variation in C3 was detected by Wieme and Demeulenaere (1967) using agarose gel electrophoresis. The polymorphism was described by Azen and Smithies (1968) and independently by Alper and Propp (1968). Azen and Smithies (1968) used high voltage starch gel electrophoresis to separate the proteins, and found 6 phenotypes, which could be attributed to four alleles. Only three phenotypes, 1, 2-1 and 2, accounted for by $C3^1$ and $C3^2$, were fairly common. Alper and Propp (1968) used prolonged agarose gel electrophoresis to detect the variations. They found 8 phenotypes, and could account for them on the basis of three certain and 2 probable additional alleles. The common variants were designated C3 F, F-S, and S, where "F" and "S" stood for "fast" and "slow". The fastest variant was called C3 F_1 and the slowest, C3 S_1. The other variants were designated according to their relative mobility taking C3 F_1 and C3 S_1 as unity. They were C3 $F_{0.8}$, $F_{0.5}$ and $S_{0.6}$. Technical refinements for C3 typing have been described (Azen *et al.*, 1969; Teisberg, 1970a). Family and population studies by a number of workers have amply confirmed the codominant inheritance pattern, and many additional variants have been described (Azen *et al.*, 1969; Teisberg, 1970b, 1971a, 1971b and 1971c; Brönnestam, 1971; Brönnestam *et al.*, 1971; Dissing and Sørensen, 1971; Segers *et al.*, 1974; Seger and Salmon, 1977; Schlesinger *et al.*, 1979). C3 S is more common in most Caucasian populations ($C3^S$ frequency about 0.8) than C3 F. The $C3^S$ allele has a very high frequency in the Lapps (Brönnestam *et al.*, 1971; Teisberg, 1971b). A possible association of $C3^F$ and rheumatoid arthritis has been suggested (Brönnestam, 1973), but no association of the C3 polymorphism with Graves disease could be detected (Pepper and Farid, 1979).

The nomenclature and recommended typing methodology for C3 is based upon agreements reached by an international panel of specialists convened at Bonn in June of 1972 (Rittner, 1973). The common C3 variants are designated C3 F and C3 S, and the responsible alleles $C3^F$ and $C3^S$, respectively. Variant types are designated according to their electrophoretic mobility on agarose gels under a specified set of conditions, with C3 S and C3 F_1 defined as having unit mobility. Thus, fast phenotypes have designations like $F_{0.8}$, $F_{1.1}$, etc., and the slow ones are similarly called $S_{0.4}$, $S_{0.65}$, etc. A heterozygote having one $C3^F$ and one $C3^{S0.4}$ gene would have the phenotype C3 FS0.4. Phenotypes representing the expression of at least 22 rarer alleles had been found by the time the Bonn conference was held. Dr. Rittner's laboratory was designated the C3 Reference Laboratory at the meeting, and an updated report of new variants was published for 1973-1974 (Rittner and Rittner, 1974). The nomenclature and other standards adopted at the June, 1972, meeting are sometimes referred to in the literature as the "Bonn conventions". C3 mobility is very sensitive to the concentration of Ca^{++} in the buffers. This cation has the effect of decreasing C3 mobility and, hence, of making the C3 bands easier to visualize by simple protein staining. The Bonn convention recommended that C3 typing be done on agarose gels in buffers containing 1.8 mM Ca lactate. Other variables in the typing procedure were discussed by Kühnl and Strobel (1974).

In 1969, Rose and Geserick observed genetic variation in a serum protein which they designated "Pt" (for "post-transferrin"). There were three phenotypes, accounted for by a pair of alleles, and the variation was seen only in aged serum samples. Before too long, it became apparent that the "Pt" system was intimately related to C3 (Geserick and Rose, 1973). Pt A is related to C3 F, Pt AB to C3 FS, and Pt B to C3 S. C3 is converted to C3a and C3b during complement activation *in vivo*, and C3b can be further converted to C3c and C3d by serum factors. It appears that C3c is a major storage fragment of C3 in serum. The so-called "Pt" system variants are apparently equivalent to the common C3 variants, which are preserved in the C3c fragments (Alper, 1973; Mauff *et al.*, 1974; Hauptmann, 1979). The conversion of C3→C3c is inhibited by 45.6 mM Na_2EDTA (Patzelt *et al.*, 1977). The common C3 variants are evident in the C3c fragment (the "Pt" system), but rarer variants cannot be readily typed using the Pt typing system. If antigen-antibody crossed electrophoretic techniques are used, C3 variants can be detected more readily in C3c fragments as well as in native C3 (Schwamborn *et al.*, 1976). C3 apparently does not cross the placental barrier, and fetal C3 corresponds to the type of the fetus and not to that of the mother (Propp and Alper, 1968; Azen *et al.*, 1969). Reviews of the C3 polymorphism have been given by Alper (1973), Seth and Seth (1976), Hobart and Lachmann (1976) and Hauptmann (1979).

The C3 polymorphism has been applied to disputed parentage problems in a number of countries (Teisberg, 1972; Farhud and Walter, 1973; Spielmann, 1973; Hoste *et al.*, 1977; Geserick *et al.*, 1980). The probability of excluding a falsely accused western European Caucasian father with C3 is about 13%. No references to C3 typing in bloodstains were found.

45.2.4 C4 Component

C4 is a glycoprotein of Mw about 206,000, consisting of

three polypeptide chains. Normal serum contains a C4 binding protein which can form stable complexes with C4. This complex formation is prevented by EDTA or heparin, however, and EDTA must be incorporated into buffers used for C4 polymorphism studies if EDTA or heparin plasma is not available (Hauptmann, 1979).

Genetic variation in C4 was described by Rosenfeld *et al.* (1969) and studied further by Bach *et al.* (1971). They studied EDTA-plasma by antigen-antibody crossed electrophoresis employing an anti-C4 serum for immunofixation detection. Ten different patterns could be distinguished, but the underlying genetics was not very clear. Teisberg *et al.* (1976) investigated C4 patterns using high voltage agarose gel electrophoresis, and immunofixation detection. They detected three common phenotypic patterns and one rarer one, and family studies indicated the involvement of three codominant alleles at an autosomal locus, which were closely linked to the *HLA* locus. The common phenotypes were C4 F, S and FS, accounted for by $C4^F$ and $C4^S$. Another phenotype, FM, was attributed to heterozygosity of $C4^F$ with a rarer allele $C4^M$. These studies were extended (Teisberg *et al.*, 1977), and an additional allele $C4^{F1}$ was found. Mauff *et al.* (1978a) confirmed these findings in a German population, and gave a modified electrophoretic procedure enabling easier detection of the F1 types. In addition to the common F, FS and S types, F1F, F1S, F1, MF, MS, MF1 and M phenotypes were observed.

Recent studies by O'Neill *et al.* (1978a, 1978b and 1978c) have resulted in a different picture of C4 genetics. It is clear that *C4* is closely linked to HLA, especially to *HLA-B* and *Bf*, but there is no clear association with *GLO* (Teisberg *et al.*, 1977; Mauff *et al.*, 1978a). The *C4* locus is thus apparently within the HLA complex. O'Neill *et al.* suggest that C4 phenotypes are controlled not by codominant alleles, but by two separate but closely linked loci. In addition, they have shown that the "red cell" antigens Chido (Ch^a) and Rodgers (Rg^a) are in fact distinct antigenic components of C4. C4 F is said to be controlled by one locus, with alleles *F* and f^0 determining the presence or absence, respectively, of the fast bands. Similarly, C4S is controlled by a second locus having alleles *S* and s^0. Ch^a is believed to be a component of C4 S, and Rg^a of C4 F, because C4 F people (FFs^0s^0 or $Ff^0s^0s^0$) are always Ch(a−), while C4 S people (f^0f^0SS or $f^0f^0Ss^0$) are always Rg(a−). The C4 antigenic determinants are associated with the C4d part of the molecule (Tilley *et al.*, 1978). Hauptmann (1979) has noted that known variations in C4 FS patterns would be consistent with the prediction of the four different genotypes for phenotype C4 FS in the two locus model. There are characteristic associations of certain HLA-B and Bf types in individuals of C4 F and C4 S type. The levels of C4 show variation according to phenotype as well, C4 F and C4 S individuals having significantly lower levels than C4 FS ones (O'Neill and Dupont, 1979). Awdeh *et al.* (1979) have recently shown that antigen-antibody crossed electrophoresis shows different and distinguishable patterns in FS, f^0S, Fs^0, FSs^0 and Ff^0S types, thus allowing their discrimination, and lending support to the two locus hypothesis. Hauptmann (1979) said that O'Neill and collaborators had recently reported finding Ch and Rg antigens on the red cells of a homozygous C4 deficient individual, but absent in the serum. This finding appears to indicate the Ch^a and Rg^a may be controlled by a locus independent of *C4*. The relationship between Ch/Rg and C4 will require further study. Hereditary C4 deficiency is quite rare, but most workers now attribute it to a rare silent allele $C4^0$ in the one locus model, or to the genotype $f^0f^0s^0s^0$ in the two locus model.

Olaisen *et al.* (1979) have recently found that the *C4* locus is duplicated, at least in some people. They suggested a change in the nomenclature for the C4 polymorphism to take this fact into account. In addition, they do not appear to regard the model proposed by O'Neill and coworkers, in which *C4* is duplicated on all chromosomes but has a high frequency of silent alleles at both loci, as proven.

45.2.5 C6 Component

Polymorphism of C6 was detected by Hobart *et al.* (1974) using isoelectric focusing and functional detection with a specific C6 hemolytic assay system. Three common patterns, called A, AB and B, could be accounted for by a pair of codominant alleles $C6^A$ and $C6^B$. Some rarer patterns were thought to be due to the expression of a less common allele, called $C6^R$. They said that $C6^R$ might not be a single allele. The common types of C6 are fairly well distributed in western Europeans, and the inheritance pattern has been confirmed (Hobart and Lachmann, 1976; Rittner *et al.*, 1979). *C6* is closely linked to *C7*, the locus controlling the seventh complement component, but it is not linked to *HLA* (Hobart and Lachmann, 1976; Kagan *et al.*, 1979). Inherited deficiency of C6 has been described, and is attributed to homozygosity for a silent allele $C6^0$ (Glass *et al.*, 1978). Ritter *et al.* (1979) and Mauff *et al.* (1979) have recommended incorporation of C6 testing into paternity investigations. The probability of excluding a falsely accused man is about 18% in western Europeans.

45.2.6 C7 Component

Hobart *et al.* (1978) found three C7 phenotypes by isoelectric focusing. There was one homozygous type, C7 1, and two apparently heterozygous types, C7 2-1 and C7 3-1. Three codominant alleles, $C7^1$, $C7^2$ and $C7^3$, were postulated to explain the types. In one family, a 2-1 × 3-1 mating had produced a 3-2 child. They noted that *C7* is closely linked to *C6*.

45.2.7 C8 Component

Raum *et al.* (1979) found genetic polymorphism in C8 using PAGIF. Two common alleles, $C8^A$ and $C8^B$, give rise to three common phenotypes. $C8^A$ is more common, having frequencies of about 0.65 in Blacks and Whites, and about 0.7 in Orientals. A rarer allele, $C8^{A1}$, is most frequent in Blacks (about 0.05), very rare in Caucasians, and it was not seen at all in Orientals. C8 deficiency is attributed to a silent ($C8^0$) allele. *C8* is not linked to *HLA*.

45.2.8 Properdin factor B (Bf)

Properdin factor B was discovered (and named) by Pillemer *et al.* (1954). Properdin is a name that has been given to several factors which can activate complement by an alternative pathway to the classical one (section 1.3.5). Thus, properdin factor B is a C3 proactivator. The protein was isolated by Haupt and Heide (1965) and called "β_2-glycoprotein II". It was also isolated by Boenisch and Alper (1970) and called "glycine-rich-β-glycoprotein" or "GBG". There was a suggestion in the work of Boenisch and Alper (1970) that the protein might exist in multiple molecular forms. Alper *et al.* (1971 and 1972) described the polymorphism of "GBG", and suggested that the protein had some relationship to the complement system. The "F", "FS" and "S" (for "fast" and "slow") types were common in all populations tested. The locus controlling the protein was named *Gb*, and the alleles Gb^F and Gb^S. A phenotype determined by a rarer allele Gb^{F1} was seen in Blacks, and one determined by another rarer allele Gb^{S1} was seen in Whites. It was then realized that GBG was identical to factor B of the properdin system (Alper *et al.*, 1973) as well as to a protein isolated by Götze and Müller-Eberhard (1971) that had been called C3 proactivator. The locus has been re-named *Bf*, and the common alleles are Bf^F and Bf^S. The less common alleles described by Alper *et al.* (1972), Gb^{F1} and Gb^{S1} are now called Bf^{F1} and $Bf^{S0.7}$. By 1978, there were seven more alleles. An internationally agreed upon nomenclature for Bf types was adopted (Mauff *et al.*, 1978b), and a Reference Laboratory headed by Prof. Dr. Mauff was set up at Cologne. Variants other than F, S and F1 are now designated "F" or "S" (depending upon whether they are "fast" or "slow") with a relative mobility value listed after the upper case letter. The reference distance in this case is S→F1. Kühnl and Spielmann (1978b) have done population studies on Bf in the German population. Weidinger *et al.* (1979) presented evidence for a silent allele, Bf^0, in one of the families in their material.

The *Bf* locus is closely linked to *HLA* (Allen, 1974; Rittner *et al.*, 1975), and it probably lies closest to *HLA-B*. There are striking linkage disequilibria between HLA-B and Bf alleles. As noted above, the *C2* and *C4* structural loci are closely associated with *Bf* in the HLA complex.

45.3 α_2–Macroglobulin (α_2M)—Xm, AL–M and α_2M

α_2-Macroglobulin is one of the major protease inhibitors of human serum (Laurell and Jeppsson, 1975), as noted in section 43.1. The molecule was purified and characterized by Hall and Roberts (1978). It had a MW of 718,000, and probably consists of 4 polypeptide chains of MW about 185,000. These smaller MW units are cleaved to yield 85,000 MW fragments upon interaction with a variety of proteases. The serum concentration of α_2M is dependent upon age and sex, mean values being of the order of 175 mg/100mℓ for men and 206 mg/100 mℓ for women. The value is 2-3 times higher in small children.

In connection with studies on Lp(a) (section 45.1.3), Berg produced an antiserum in rabbits which, after absorption, reacted with some but not all human sera. The precipitates stained for protein, but not for lipid, and the antigen was, therefore, not a lipoprotein. The antiserum had been produced by immunization with the serum of a single Lp(a–) female donor. Reactions of the antiserum strongly indicated that the antigen might be controlled by an X-linked gene. The antigen could also be localized to the macroglobulins, and the system was named "Xm" to indicate the X-linked macroglobulin protein antigen. The antigen was called Xm(a) and the gene controlling its presence Xm^a (Berg and Bearn, 1966a and 1966b). The antigen was shown to be localized in α_2M, and extensive linkage studies were carried out with other known X markers (Berg and Bearn, 1968). *Xm* mapped fairly near "deutan" and a little farther from *Gd*. Cooper (1978) said that the limited supply of anti-Xm(a) reagent had been exhausted, and that no further examples had been found.

In 1969, Kasukawa *et al.* found an isoprecipitin in a chronic hepatitis patient who had never been transfused. It reacted with about 30% of random sera in Japan, and the antigen, which was said to be an α_1-globulin, seemed to be inherited in a simple Mendelian way. Another example of the antiserum was then found by Leikola *et al.* (1972), and they showed that the antigen resided in the α_2M fraction. The antigen was named "AL-M", and it was inherited as an autosomal dominant.

In 1974, Gallango and Castillo reported another polymorphism in α_2M. This one was detected by immunoelectrophoresis in the sera of a Venezuelan Mestizo population using anti-α_2M sera. The types were distinguished by electrophoretic mobility upon immunoelectrophoresis. There were apparently three homozygous types, A, B and C, and three heterozygous types AB, BC and AC. Genetic data indicated that the types could be accounted for by three codominant alleles at an autosomal locus, α_2M^A, α_2M^B and α_2M^C. The autosomal inheritance pattern indicated nonidentity with the Xm system. Identity with AL-M seemed to be excluded by the fact that no "negatives" had been observed. Mroueh and Adham (1970) assayed semen for α_2M levels, and found measurable quantities in 11 out of 34 samples. Concentrations in the 11 ranged from 30 to 130 mg/100mℓ with a mean of 70.4. There was no correlation between α_2M level and oligo- or azoospermia.

45.4 α_1–Acid Glycoprotein (Orosomucoid)

α_1-Acid glycoprotein is a plasma globulin characterized by a high carbohydrate content, a large number of sialyl residues and a very acidic pI. The protein occurs in the so-called "seromucoid" fraction of plasma, and is the fastest moving serum protein component on starch gel electrophoresis using borate buffers at pH 8.6. One of the first purified preparations of the protein was described by Weimer *et al.* (1950). At that time, it was referred to as the "MP-1 component". Schmid (1953) obtained a crystalline preparation

of α_1-acid glycoprotein. The purified protein had a MW of about 40,000, and about 45% of its weight is attributable to the carbohydrate content. The primary structure of α_1-acid glycoprotein has been completely solved (Schmid *et al.*, 1973), and upon close analysis, revealed an extraordinary number of amino acid substitutions in different examples of the protein. 21 different residues, out of 181 in the complete molecule, have been found to be substituted. The sequence also showed structural homology with the immunoglobulins.

In 1964, Schmid *et al.* found that α_1-acid glycoprotein exhibited electrophoretic heterogeneity, and suggested that this behavior might have a genetic basis. Further studies were carried out on desialyzed material, and indicated that the three different patterns which could be observed were the result of a pair of alleles (Schmid *et al.*, 1965). Because of its molecular properties, the protein shows considerable microheterogeneity upon electrophoresis, in addition to the macro polymorphic patterns. This microheterogeneity is even more apparent if isoelectric focusing is used as the separation technique (Gordon and Dykes, 1972).

The α_1-acid glycoprotein phenotypes were studied by immunofixation electrophoresis on agarose gels by Johnson *et al.* (1969). Further family and population studies, extending the work of Schmid *et al.* (1965), were conducted to establish the genetic basis for the polymorphism. The phenotypes were called SS, SF and FF (where "S" and "F" stand for "slow" and "fast"). These are accounted for by a pair of autosomal codominant alleles called Or^S and Or^F (the protein is also called "orosomucoid"). The Or^S frequency in U.S. Caucasians (n = 220) was 0.36, and varied from about 0.33 to 0.57 in other populations. The polymorphism is thus fairly well distributed, and may well become another valuable serum group system marker.

Sayce and Rees (1977) reported a series of experiments on seminal orosomucoid. There appeared to be two different and distinguishable immunological reactions using anti-orosomucoid serum. These experiments weere discussed briefly in section 10.13.2. The relationship between these immunologically distinguishable forms and the serum polymorphic types, if there is any relationship, is not clear.

The properties of α_1-acid glycoprotein have been discussed in detail by Schmid (1975). The biological role of the protein is not yet fully clear.

45.5 Ceruloplasmin (Cp)

Copper is an essential trace element in the nutrition of many animals, including human beings. Most of the copper in human serum is bound to an α_2 globulin protein (Holmberg and Laurell, 1947). Cp has a MW of about 150,000 and can bind 8 atoms of Cu per mole. The isolated protein has a strikingly beautiful sky blue color, which is often mentioned in discussions of it. The name "ceruloplasmin" was given to the protein by Holmberg and Laurell (1948). Cp has been extensively purified, and a crystalline preparation was obtained by Deutsch (1960). The detailed properties of the protein are described in the review of Poulik and Weiss (1975). The detailed structure is apparently still not completely clear. Subunit structure has been proposed for Cp (see in Poulik, 1968), but there is also evidence to suggest that the molecule consists of a single polypeptide chain (Poulik and Weiss, 1975). Kingston *et al.* (1979) determined the sequence of a lengthy segment of the molecule (159 amino acid residues), and appeared to favor a single chain structure for the intact protein. This segment did not contain any of the carbohydrate.

The exact function of Cp is still not settled altogether. The protein exhibits oxidase activity with a number of artificial substrates. Cp is usually detected by its oxidase activity after electrophoretic separation. Uriel (1958) devised such a detection procedure with p-phenylenediamine as substrate. The protein is also an effective "ferroxidase", catalyzing the conversion of Fe^{2+} to Fe^{3+}. It has been assigned a name and number by the Enzyme Commission on this basis: ceruloplasmin = ferroxidase; iron(II):oxygen oxidoreductase; E.C. 1.16.3.1. The ferroxidase activity is one of the three functions of Cp that have been assigned physiological significance, the others being copper transport and detoxication, and the maintenance of copper homeostasis in tissues. The functions are not necessarily mutually exclusive. At the present time, a number of investigators think that the ferroxidase activity of Cp is its most important function. Ferrous ions are oxidized by Cp to ferric ions, which are then incorporated into transferrin (section 42). Goldstein *et al.* (1979) have shown that Cp can also act as a scavenger of superoxide radicals, thus mimicking the action of SOD (section 37.4) in this respect.

Molecular heterogeneity was observed in Cp by a number of the earlier workers, such as Uriel (1958), Deutsch (1960), Morell and Scheinberg (1960) and Hirschman *et al.* (1961). A genetic basis for the variation was considered, but Poulik and Bearn (1962) indicated that it would be difficult to interpret the genetic significance of the heterogeneity. Other explanations, such as polymerization and denaturation caused by methods of preparation or storage, were considered more likely.

Qualitative genetic variation in Cp is now known, however. A possible genetic variant was described by Martin *et al.* (1961) and more fully by McAlister *et al.* (1961). The variant form was detected by vertical starch gel electrophoresis at pH 8.6. It was faster than the usual Cp (called "Cp 1") and was called Cp 1-F. It was easier to detect, they said, in pH 9.1 buffers. The variant Cp was transmitted through four generations of a family as a dominant trait. In spite of large scale Cp typing in a number of laboratories, however, this variant was not encountered again (Poulik and Weiss, 1975).

Schreffler *et al.* (1971) described three genetically determined forms of ceruloplasmin, called Cp "A" (the fastest), "B" (intermediate) and "C" (the slowest), which could occur alone, or in combinations. Five phenotypes were seen: A, AB, B, AC and BC. These phenotypes were attributable to three codominant alleles at an autosomal locus, Cp^A, Cp^B and Cp^C. Cp B is the common phenotype. Cp^A oc-

curred in Caucasians but was more frequent in Negroes, while Cp^C was not seen in Caucasians. These phenotypes were detected by starch gel electrophoresis in borate buffers, pH 9. Shokeir and Schreffler (1970) described two additional variants in the sera of American Blacks, Cp New Haven (Cp NH) and Cp Bridgeport (Cp Bpt). Cp NH migrated between Cp B and Cp C, and was due to an allele of Cp^A and Cp^B called Cp^{NH}. Cp Bpt was seen in a Black family, and migrated between Cp A and Cp B. Its genetic relationship to the other forms was not as clear. Cp^{NH} had a higher frequency in American Blacks than did Cp^C. Shokeir (1971) compared the biochemical and immunological properties of Cp A, AB, AC, ANH, BNH and BptB. All the forms were of similar molecular size, and charge differences were not attributable to differences in sialic acid content. No immunological differences were detected with an anti-human Cp, but there were some differences in the inhibition of oxidase activity by cyanide or azide. McCombs *et al.* (1970) have described another variant, Cp Galveston, detected by slab polyacrylamide gel electrophoresis in pH 9.1 buffers. The variant protein was seen in serum and in ascites fluid, and although an additional allele was suspected, family studies could not be carried out.

One, and possibly two, new variants were described by Buettner-Janusch *et al.* (1973) in the Malagasy population of Madagascar. They found that Cp B was the common phenotype, as in other populations. Examples of Cp NH, Bpt and Galveston containing phenotypes were observed among the 405 sera. A previously undescribed phenotype, called Cp 'Tananarive' (or Cp 'Tan') was observed as A/Tan and Tan/Tan in this population. No B/Tan heterozygotes were seen. The failure to find B/Tan types might mean that the allele determining 'Tan' was not codominant with Cp^B, but it could also mean that the sample of the population typed simply did not include a person of this type. Sera were observed that typed as 'Cp O' by normal testing procedures. If these were subjected to prolonged activity staining, however, a weak band, which was named 'Cp X', eventually appeared. The band appeared to represent Cp and not some other plasma protein, but its relationship to the other Cp variants was not completely clear.

Genetically controlled quantitative variation in Cp is known, the most noted manifestation of it being Wilson's disease. The first complete description of this comparatively rare disorder was given by Wilson in 1912. The disease has been called hepatolenticular degeneration, since it is characterized in adults by deterioration of the cerebral lenticular nuclei and by progressive cirrhosis of the liver. It is also characterized by greatly decreased levels of serum Cp (hypoceruloplasminemia). The major biochemical feature in Wilson's disease is the disruption of normal copper metabolism. The defect is inherited as an autosomal recessive. Wilson's disease patients have been studied quite extensively, but a full understanding of the molecular pathology does not appear to have emerged as yet. Gitlin and Gitlin (1975) and Poulik and Weiss (1975) discussed the subject in detail. There has been an inclination to regard the primary biochemical lesion as the inability to synthesize Cp, but evidence is conflicting. Studies on "Wilsonian" Cp as compared to that from normal individuals (e.g. Neifakh *et al.*, 1972) have not yielded a clear set of differences. Some have suggested that the disease may result from homozygosity for different genes in different populations, or perhaps from different modifying genes.

45.6 Transcobalamin II (Tc II)

Vitamin B_{12} binding and transport in serum are mediated by two types of transcobalamins, called I and II. Tc I is about three times larger in MW than Tc II (Hall, 1969). Tc II has been purified from human serum and characterized to some extent. Allen and Majerus (1972) obtained an affinity purified Tc II that had a MW of 53,900 by ultracentrifugation, and bound one Vitamin B_{12} per mole of protein. It dissociated in SDS to yield two peptides, and did not appear to contain carbohydrate. Lindemans *et al.* (1979) also purified Tc II by affinity chromatography on cyanocobalamin-Sepharose. The MW of the native molecule was about 37,000, but a faint band of MW about 29,000 could also be seen on SDS-polyacrylamide gel electrophoresis. The preparation was heterogeneous by isoelectric focusing. Haus *et al.* (1979) have found that human Tc II seems to be distinctly different immunologically from the protein in other species.

In 1975, Daiger *et al.* found polymorphism in Tc II by polyacrylamide gel electrophoresis and autoradiographic detection using radiolabelled Vitamin B_{12} (Daiger *et al.*, 1975b). There were some 7 different patterns, with 2–4 bands each. They were stable in a given person and inherited. Four alleles at an autosomal locus were postulated to explain the types. Tc^3 and Tc^4 were quite common, while Tc^1 and Tc^2 were rare. Tc^1 bands were the fastest migrating, and Tc^4 bands, the slowest. Heparinized plasma gave very poor results, perhaps because heparin binds these proteins. Similar observations on the polymorphism have been made by Fràter-Schröder and Hitzig (1977) and Fràter-Schröder *et al.* (1979a and 1979b). They too used polyacrylamide gel electrophoresis and radiolabelled vitamin B_{12} autoradiography for typing, and could distinguish eleven phenotypes, three of which were fairly common. These were called Tc II 3-3, 1-3 and 1-1. They were accounted for by two common alleles $TcII^1$ and $TcII^3$ at an autosomal locus. Three additional alleles, $TcII^2$, and $TcII^4$ and $TcII^5$ had lower frequencies. Family studies were consistent with the five codominant allele explanation. Occasional families with an inherited Tc II deficiency have been described (Hitzig *et al.*, 1974), and deficient children usually suffer from megaloblastic anemia. Two unusual Tc II patterns in patients with anemias have been described as well (Fràter-Schröder *et al.*, 1979a)

45.7 Thyroxine Binding α–Globulin (TBG)

Throxine in human plasma may be bound to three different proteins: thyroxine-binding prealbumin (TBPA),

thyroxine-binding globulin (TBG), and albumin. TBPA was first isolated by Schultze *et al.* (1962d) and designated "tryptophan-rich prealbumin" because its electrophoretic mobility was faster than that of albumin at pH 8.6. The protein has since been more thoroughly characterized. The amino acid sequence has been worked out (Kanda *et al.*, 1974), and there are only two Try residues per polypeptide chain of 127 amino acids. The x-ray structure of TBPA at a resolution of 2.5Å has also been published (Blake *et al.*, 1974). TBPA can bind the retinol-binding protein (RBP), the specific vitamin A carrier in plasma, on a one for one mole basis. The TBPA exists in a tetrameric structure for the binding of RBP or thyroxine, and complexation with TBP apparently has no effect on thyroxine binding (Putnam, 1975b; Cooper, 1978). Characteristics of retinol-binding protein are further discussed by Putnam (1975b). The majority of protein-bound thyroxine appears to be associated with TBG, the thyroxine-binding capacity of TBPA apparently being secondary to its RBP-binding function (Cooper, 1978). Thyroxine binding to albumin is nonspecific, and apparently not of much physiologic significance (Putnam, 1975b).

TBG has not been characterized as well as TBPA, in part because the former is present in serum at significantly lower concentrations (1–2 mg/100mℓ) than that of the latter (10–40 mg/100 mℓ) (Putnam, 1975b). Giorgio and Tabachnik (1968) developed a purification scheme for TBG from human plasma, resulting in a preparation that was homogeneous by starch gel and polyacylamide gel electrophoresis as well as by ultracentrifugation. The purified glycoprotein had a MW of 58,000.

Familial quantitative variation in TBG has been reported. Nicoloff *et al.* (1964) first noticed the near absence of the protein in six members of a family whose members had no evidence of Thyroid disease. Nikolai and Seal (1966 and 1967) observed TBG deficiency in a second family, and could show that the characteristic was X-linked. Other pedigrees showing this same behavior have been noted, and in one family the TBG deficiency was linked to the Xg^a blood group (Grant *et al.*, 1974). Shane *et al.* (1971) found an inherited elevated level of TBG in four generations of a family. The characteristic was X-linked, and other families are apparently known which show it as well.

Daiger (1976) described an electrophoretic variant of TBG in Black and Oriental populations. The variant gene occured in about 15% of American Blacks. The variant locus is apparently X-linked as well, but it is not clear whether it is identical to the locus controlling the quantitative variations.

45.8 Albumin

Serum albumin is the most familiar and most plentiful of the serum proteins. Serum normally contains 42 ± 3.5 g/ℓ of albumin (with a range of 35–50. Albumin represents about 60% of the total serum protein. It has been assigned numerous functions, including maintenance of the osmotic pressure of blood, fatty acid transport and sequestration, and transport of bilirubin. Albumin was extensively reviewed by Peters (1975).

Serum albumin has been extensively purified and characterized. It is a simple protein, containing one polypeptide chain and no carbohydrate. Some fatty acid is apparently associated with even the purest examples of the protein. Human albumin has a MW of 66,248 (calculated from the amino acid composition). Its sequence has been completely determined (Brown, 1975), as has that of the bovine protein (Behrens *et al.*, 1975). Both sequences may also be found in Peters (1975). There are 17 intrachain disulfide bridges, creating a structure with 9 loops. Albumin is well known for its ability to associate with a great number and variety of small molecules, including cations, anions (especially fatty acid, but including hormone and amino acid), drugs and organic dyes. Albumin is a good antigen, and anti-albumin is surely a major constituent of the "anti-human serum" prepared in animals (section 16.1). Atassi *et al.* (1979) have recently reported that the bovine protein has five antigenic sites.

Strictly speaking, albumin is not polymorphic in most human populations. It does exhibit a large number of structural variants, most of which are exceedingly rare. A few variants reach significant frequencies in isolated populations. Albumin variants have been reviewed at length by Gitlin and Gitlin (1975). Structural variants are of two types. One of these is represented by the group that is said to give rise to "paralbuminemia", "bisalbuminemia", or "alloalbuminemia". The terms are essentially equivalent in most of the literature, and mean simply that more than one albumin band (usually two) is seen upon electrophoretic separation. The other type is represented by albumins which tend to dimerize. Quantitative variation is known, and is represented by markedly low albumin levels, usually termed "analbuminemia".

Genetic variation was first noted by Nennstiel and Becht (1957) and by Knedel (1958). They observed two albumins in serum by electrophoresis, undoubtedly the reflection of heterozygosity for a variant structural gene. A large number of variants have since been described, especially by Weitkamp and collaborators. Normal albumin (the usual kind) is often called "A". Many others are named after places. Until the variants can be studied structurally, it will be difficult to know exactly which ones are really different. Weitkamp *et al.* (1969) described eight distinguishable "types" of albumin, but they said that each "type" might be a collection of a number of different variants. In 1973, Weitkamp *et al.* carried out extensive studies on 23 variants. The most frequent variant among Europeans is called "B", but even it is quite uncommon. Weitkamp *et al.* (1973) said that the frequency of albumin variants may be as low as 1 in 2,000 in Europe. Several electrophoretic typing systems must be used to distinguish all the different variants. Fine *et al.* (1976) examined 24 cases of albumin variants in France, and 16 of them were type B. Efremov and Braend (1964) saw only one variant in 1,015 Norwegians, and no variants were found by

Persson *et al.* (1971) in Greenland Eskimos. Some albumin variants have been found only in isolated populations (Lie-Injo *et al.*, 1971; Frohlich *et al.*, 1978). People with familial analbuminemia have been described (Bennhold and Kallee, 1959), but are very rare. Apart from the family studies by Bennhold and Kallee (1959), Gitlin and Gitlin (1975) said that only 10 additional cases had been seen. It is not clear what the exact nature of the defect is in these cases, since "analbuminemics" do make a small amount of albumin. In addition, the known cases may not all have the same genetic basis. The albumin locus is known to be linked to *Gc* (Weitkamp *et al.*, 1966 and 1970).

Since albumin is not significantly polymorphic in most populations, it does not provide a useful genetic marker system for medicolegal problems. Nevertheless, Reinskou (1968b) reported that one of two putative fathers in a paternity case had an albumin variant which had been inherited by the child. The man had also passed a Gc^2 gene to the child. The case illustrates the value of rarities when they do occur in a case, and are detected.

45.9 β_2–Glycoprotein I (Bg)

This protein was isolated from human plasma by Schultze *et al.* (1961). It may be recalled in passing that this group of investigators isolated a "β_2-glycoprotein II" (Haupt and Heide, 1965), which was later recognized as being identical with factor B of the properdin system (section 45.2.8). Heimberger *et al.* (1964) described a number of the characteristics of the molecule. Liu and Putnam (1975) have reported similar studies more recently. The MW is about 40,000, and the molecule appears to be a single polypeptide chain about 300 amino acids long with 8 or 9 disulfide bridges. It occurs in normal plasma at concentrations of 15–30 mg/100 mℓ. It has no known function.

In 1968, Haupt *et al.* found a family in which two sibs had no β_2-glycoprotein I and whose parents had about half the normal amount. Cleve (1968) then carried out a population study using radial immunodiffusion technique. 94% of people had concentrations ranging from 16–30 mg/100 mℓ with a mean of 21, while the remaining 6% had concentrations of 6–14 mg/100 mℓ with a mean of 10 in their serum. The distribution was sharply bimodal in these initial data, and it was proposed that β_2-glycoprotein I concentration was controlled by a pair of codominant alleles called Bg^N and Bg^D at an autosomal locus. Bg^NBg^N people have the higher concentration range, while Bg^NBg^D people have the lower one. "N" and "D" stood for "normal" and "deficient". The two sibs of Haupt *et al.* (1968) with no detectable protein were presumably uncommon Bg^DBg^D homozygotes. Bg^D is thus probably a silent allele. Various nongenetic factors like sex, age, pregnancy and disease affect the concentration, however. One family in the material studied by Cleve (1968) did not fit the model. Cleve and Rittner (1969) studied 88 families with 213 children for this characteristic. In 9 families, one parent and about half the children appeared to be Bg^NBg^D. But a few families did not fit the model. Parents with normal serum concentrations can have children who have intermediate levels. Koppe *et al.* (1970) studied an additional 49 families, and again, a few exceptions were found. Atkin and Rundle (1974) looked at 381 people in the British population, and found almost the same Bg^N frequency as in the German population (about 0.94). They also found one deficient person. It is assumed by most investigators that the basic genetic hypothesis is correct, and that most of the variation is controlled by a single *Bg* locus. The situation resembles that of Lp^a in some ways (section 45.1.3), except that the codominant nature of the *Bg* alleles, in contrast to the dominance of Lp^a, makes the variation somewhat easier to interpret (Cooper, 1978).

SECTION 46. THE HLA SYSTEM AND THE HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX

46.1 Introduction

In his Nobel address in 1930, Landsteiner drew a parallel between hemolytic reactions caused by the transfusion of blood-group incompatible blood and skin graft rejections. The two phenomena, he said, ". . . .are basically related and rest on chemical differences of a similar kind".

The immunological system is fairly similar in all vertebrates, and vertebrates reject foreign skin transplants as well as react against foreign particles or fluids by the formation of specific antibodies. Rejection of "foreign" skin grafts may be acute (within weeks), or chronic (within a few weeks or months). In species able to reject acutely, it has been possible to identify a small region on a single chromosome in which numerous genes controlling immune reactions are located at closely associated loci. This genetic region is called the "major histocompatibility region", or "system", or "complex" (MHR, MHS, MHC). Mammals are included among those vertebrates having a MHC. In animals capable only of chronic rejection, the responsible genes are distributed throughout the genome. Even in acutely rejecting species, which have a MHC, however, there are additional genetic loci whose products are associated with chronic rejection, and these are distributed throughout the genome.

The pioneering work in the recognition of mammalian major histocompatibility regions was done by Gorer and Snell and their collaborators beginning in the 1930's. Their work was with mice, and resulted in the definition of the H-2, or major histocompatibility system, in the mouse. Major histocompatibility complexes are now recognized in the mouse, rat, chicken, guinea pig, dog, rhesus monkey, chimpanzee and in human beings (Albert and Scholz, 1978; Götze, 1977). The H-2 system in the mouse has been an important model in many respects for the studies responsible for unravelling the MHC in humans.

In 1952, Dausset and Nenna found antibodies in a multiply transfused patient which agglutinated the leucocytes of some, but not all other people. The presence of leucoagglutinins in multiply transfused people was found to be a more general occurrence (Dausset, 1954). In 1958, Dausset described the first antigen in what was to become the HLA system. It was called "Mac" (today, HLA-A2). A second leucocyte polymorphism was found by van Rood (1962). He called the responsible locus "4" (or "FOUR"), and the two supposed alleles "4a" and "4b". Recognition of these specificities was achieved primarily by statistical genetic analysis of many antibody-containing sera which had been tested against panels of leucocytes from selected, unrelated donors. Using similar methods, Payne *et al.* (1964) described another polymorphism independent of FOUR. It was called "LA" (where "L" stood for "leukocyte" and "A" was supposed to indicate the first locus). From these beginnings, an understanding of the HLA system has emerged over the past 15 or 20 years. The evolution of developments in this field has been characterized by an impressive and very productive degree of international cooperation and agreement. In 1980, Dausset and Snell shared the Nobel Prize for Physiology or Medicine with Benacerraf, for their work on the "genetically determined structures of the cell surface that regulate immunological reactions" (Marx, 1980). Gorer, who died in 1961, would probably have been included in the group, had he lived.

The HLA and genetically associated systems constitute the most intricate and complex array of polymorphisms yet described. The impressive degree of international cooperation and agreement that has characterized the developments in HLA serology has surely simplified the nomenclature problems, and provided order in what could otherwise have been a chaotic nightmare of complicated data. Even given the relatively organized history of the system, the complexities of trivial nomenclature, workshop nomenclature and official nomenclature, along with the recognition of such a large number of different specificities, can be quite bewildering to the nonspecialist. Many of the antisera contain antibodies to multiple specificities, others contain cross reacting antibodies, and it has taken time to recognize and understand all these facts. Not all the specificities are detected by the same methods either. Several different methods have been used to recognize and type the lymphocyte-defined antigens especially, and the different methods do not always give exactly concordant results. The sheer extent of the HLA system, however, makes this system the best genetic marker system yet described. This value has already been exploited in parentage investigations, but there is little work as yet on the determination of HLA antigens in dried blood.

No review as brief as this one can do justice to the history, development and complexities of HLA. Many excellent reviews are available, however, including those of Mayr (1969), Walford (1969), Amos and Ward (1975), Albert (1976), Albert and Götze (1977), Götze (1977), Miller (1977), Albert and Scholz (1978), Bodmer (1978a), Bender (1979) and Perkins (1979). Beginning in 1964, a series of eight international conference/workshops has been held periodically, at which the specialists have exchanged information and reagents, and have reached agreements about the status of the HLA system. A nomenclature committee made up of internationally recognized specialists, and functioning under

the auspices of the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS), has met after the last few of these conferences, and set forth the current standardized nomenclature. The proceedings of the eight conferences, held at Durham, Leiden, Torino, Los Angeles, Evian, Aarhus, Oxford and again in Los Angeles, (Histocompatibility Testing, 1964, 1965, 1967, 1970, 1972, 1975, 1977 and 1980) provide an accurate picture of the prevailing information about HLA during its evolution and up to the present time.

46.2 Definition and Function of HLA Specificities

Detailed genetic characterization of the H-2 region in the mouse has come about largely through the use of inbred strains of animals developed for the purpose by the pioneering investigators. Human populations are, however, highly outbred, and contain a large number of haplotypes representing a large number of recombinations. Accordingly, population and statistical genetic methods have played a major role in the analysis of the human serological data. Thus, although there are clear analogies between human and mouse MHC genetics, different methods have been required to arrive at a detailed understanding of them.

It became clear early in the development of HLA serology that leukoagglutinating, and later cytotoxic, antibodies are found in the sera of pregnant and parous women (Payne and Rolfs, 1958; van Rood *et al.*, 1958). A substantial percentage of pregnant or parous women turn out to have in their sera lymphocytotoxic antibodies against the HLA antigens of the child which were inherited from the father (Wolf, 1971; Escolar and Mueller-Eckhardt, 1971; Nymand *et al.*, 1971; Bertrams *et al.*, 1971). In the first years of HLA research, it was not possible to find two antisera which gave exactly identical reaction patterns against defined panels of donor cells. This behavior was recognized as being indicative of an extraordinary antigen diversity and structural complexity, but it also made the task of sorting out the specificities more difficult. At the beginning, large numbers of cell samples form unrelated donors were tested against large numbers of different undefined sera. The resulting reaction patterns were then analyzed by a complex series of 2×2 comparisons of pairs of antisera, in which each antiserum is eventually compared with all others. These analyses, done in a number of different laboratories, were made more manageable with the help of computers. Details of the 2×2 comparison method will be found in Cavalli-Sforza and Bodmer (1971). Briefly, such an analysis is designed to show similarity or dissimilarity between members of a pair of antisera, and combinations of these analyses leads to definitions of groups of sera which are highly associated. Associations between sera indicate that they share antibodies directed against the same antigen. Cell samples that react with all the sera of a highly associated group are inferred to possess the "antigen" corresponding to the "antibody" common to all the sera in the group. In this way "antigens" can be defined even when the only antisera against them are polyspecific, although these definitions are operational. Operationally defined antigens have frequently been found to be of a compound nature as more refined analysis with more and different antisera, and different cell panels, became possible. The revisions in the HLA nomenclature over the years largely reflect these changes in the operational definitions of the antigens.

The involvement of cell surface antigens in transplant rejections was clearly shown in animals, and these antigens were known to be present on a variety of tissues and on leucocytes. van Rood *et al.* (1964) and Dausset *et al.* (1965) carried out studies on the relationship of the leukocyte antigens to skin transplantation survival. These studies have been extended by van Rood *et al.* (1966) and by many others, and it was soon clear that HLA represents the major histocompatibility complex in the human species (Bach and Amos, 1967).

46.3 Genetics and Nomenclature of the Serologically Defined HLA Loci

46.3.1 HLA-A and HLA-B Antigens

As noted in 46.1 above, it was clear, at least by 1964, that there appeared to be two different genetic loci controlling the lymphocyte surface antigens. These were originally called "4" ("FOUR") and "LA" and both were multiple allelic. The antigens controlled by the multiple alleles were inherited in a simple Mendelian codominant fashion (e.g. Payne and Hankel, 1961). Dausset *et al.* (1965) proposed a unifying genetic model in which these two loci were seen as part of the same system (called "Hu-1", but later known as "HLA"). Population and family studies indicated that these two loci were autosomal and closely linked (Dausset *et al.*, 1965; Bodmer *et al.*, 1966; Ceppellini *et al.*, 1967). The first unequivocal report of crossing over between the *LA* and *FOUR* locus genes was that of Kissmeyer-Nielsen *et al.* (1969).

The Nomenclature Committee in 1967 combined the FOUR and LA loci into a system that was called "HL-A". The different alleles were assigned numbers, in the sequence of their discovery or of their final definition. Rapid developments in the field resulted in a large number of newly discovered antigens, and of antigens whose status was not immediately clear. In 1970, an important new agreement was reached. Most of the newly discovered antigens, which had a variety of local and unsystematic designations that were quite confusing, were given provisional HL-A numerical designations. The provisional designation was indicated by a "w" (or "W"), which stood for "workshop", according to Bender (1979). The "w" designations were chosen so that the conversion to a recognized specificity could be accomplished simply by omitting the "w". In 1976, the Nomenclature Committee set forth the rules which, with minor modification, still form the basis of HLA nomenclature (WHO-IUIS Terminology Committee, 1976). The system was to be called "HLA" (the dash in HL-A was dropped). The genetic loci of the system would be designated by upper case letters, A, B, C, D, etc., as necessary.

The "A" locus would thus be called "HLA-A", the "B" locus "HLA-B", and so on. Antigens would continue to be designated by numbers, assigned by international agreement. Provisional specificities would continue to be indicated by the prefix "w" or "W". These would continue to be assigned so that "promotion" to a fully recognized specificity could be achieved by dropping the "w". A lower case "w" was preferred for the provisional designation, but the upper case "W" was permitted because it was realized that most computer output devices are incapable of printing lower case letters. The loci previously called "LA" (first locus) and "FOUR" (second locus) would henceforth be called 'HLA-A" and "HLA-B", respectively. For historical reasons, specificities belonging to HLA-A and HLA-B are numbered jointly, so that there is no overlap in numbers between them. Thus there are antigens HLA-A1, HLA-A2 and HLA-A3, but there are no "HLA-B1", "HLA-B2" or "HLA-B3", and so forth. There are certain criteria for the recognition of specificities, and for the recognition of provisional specificities. These have been somewhat refined as time has gone along. Each major workshop has resulted in the recognition of some new provisional specificities, and the conversion of previously provisional specificities to fully recognized ones.

By the time of the sixth workshop in Aarhus in 1975 (Histocompatibility Testing, 1975), there were 8 fully recognized HLA-A specificities and 12 additional "w" or provisional ones. There were eight specificities at HLA-B and 12 provisional ones (WHO-IUIS Terminology Committee, 1976). In the next report (WHO-IUIS Nomenclature Committee, 1978) 10 established and 10 provisional specificities at HLA-A, and 12 established and 21 provisional ones at HLA-B were listed. Most recently (WHO-IUIS Nomenclature Committee, 1980), there were no changes in the HLA-A locus antigens. At HLA-B, there were nine new provisional specificities, HLA-Bw55 through Bw63.

It should be noted that the HLA-A, HLA-B and HLA-C (see below) antigens are defined principally by serological reactions, and are sometimes referred to as "SD" ("serologically detected" or "serologically defined") antigens. The loci have been called *SD1*, *SD2*, and *SD3* at times. In section 46.4 we will briefly discuss HLA antigens that were defined originally by other than serological reactions.

46.3.2 Cross reactivity of antisera and "splits"

The phenomenon usually called cross reactivity (the term is not altogether accurate according to Albert and Götze, 1977) has been a major problem in fully understanding HLA serology. Cross reactivity was recognized by Svejgaard and Kissmeyer-Nielsen in 1968, and is a fairly general phenomenon among certain combinations of HLA antigens. When immunization experiments were done in which the donor differed from the recipient for only one known HLA antigen, it was observed that the resulting antiserum reacted not only with cells positive for the antigen that was different (and to which the antibodies were expected to be directed), but also with cells positive for other antigens. For example, an antiserum produced by immunizing an HLA-A2,A3,B8,B12 recipient with HLA-A2,A3,B7,B12 cells reacted with all HLA-B7 positive cells (as expected), but also reacted with cells positive for HLA-B27 or Bw22, even though the immunizing cells did not carry these antigens. Absorption of this antiserum with cells positive for HLA-B27 or Bw22, but negative for B7, removed all the activity. It was thought for a time that this phenomenon was due to overall biochemical similarity of the antigens, but the idea is contradicted by the existence of anti-HLA-B7 sera which cannot be absorbed by HLA-B27 or Bw22 cells. This kind of cross reactivity was found to be a generalized phenomenon among certain groups of antigens, e.g. HLA-A1, A3, A11, or HLA-A2, A28 at the HLA-A locus, and HLA-B7, B27, Bw22, or HLA-B5, Bw35, Bw15, B18 at the HLA-B locus. The patterns of cross reactivity were seen not only in antiserums resulting from planned immunizations, but in those obtained from unselected multiparous women as well. The groups of cross reacting antigens, it may be noted, always involve the products of the same locus. Titration experiments with antisera directed at a group of cross reacting antigens indicate that most of them have one "main" specificity that is relatively high titered in comparison with the activity toward the other specificities. This behavior has led to the subdivision of some antigenic specificities into what Bender (1979) called "subtypes". The terms "broad" and "narrow" specificity are also used in this context. Thus, HLA-Aw23 and Aw24 are regarded as "subtypes" of HLA-A9. Another way of putting it would be to say that A9 is the "broad" specificity, while Aw23 or Aw24 are "narrow" ones. Similarly, HLA-A25 and A26 are subtypes of HLA-A10. The divisions of HLA-A9 into Aw23 and Aw24, or of A10 into A25 and A26, are referred to in the literature as "splits", and there are quite a number of them known at HLA-A and HLA-B. The nomenclature rules allow for specification of the broad specificity in parentheses next to a narrow one, e.g. HLA-Aw23(9).

46.3.3 Formal genetics and chromosomal localization

Many population and family studies have been carried out on the HLA system (e.g. Svejgaard *et al.*, 1970; Mayr, 1971 and 1977; Colombani and Degos, 1972; Spielmann *et al.*, 1974; Greenacre and Degos, 1977). A child inherits an HLA *haplotype* from each parent. The haplotype is, essentially, the series of HLA genes on the chromosome that is inherited, and consists of an HLA-A allele, an HLA-B allele, and one allele each from the additional loci to be discussed below. The sum of the two inherited haplotypes is the individual's genotype, although it is not always possible to discern the genotype from the phenotype, since one phenotype may be dictated by a number of different genotypes. If the paternal chromosomes are designated P/P′ and the maternal ones M/M′, four types of children are possible: P/M, P/M′, P′/M and P′/M′, where P, P′, M and M′ represent haplotypes (or chromosomes). If the HLA-A, -B and -C loci are all considered, up to six different alleles could occur in a person, e.g. HLA-A2,A3,B12,Bw35,Cw4,

Cw5, representing the haplotypes HLA-A2, B12,Cw5 and HLA-A3,Bw35,Cw4. Haplotypes cannot be determined from phenotypes without doing family studies. Typing results often yield information about five, four or even fewer alleles, and it is not possible to distinguish between homozygosity for an allele, and the presence of a serologically undefined allele (the latter often called a "blank"). In a case, for example, of an HLA-A2,A9,B12 phenotype, the genotype could be A9,B12/A2,B12 or A9,B12/A2,BX or A9,BX/A2,B12, where BX represents a serologically undefined allele at HLA-B. The probable genotype of the person could be calculated on the basis of haplotype frequencies. The situation is reminiscent of Rh system genetics (section 22). Suppose the HLA-A2,A9,B12 person in the example above were a mother with three children, all of whom had inherited an HLA-A9,B12 haplotype; this result would show that she had an HLA-A9,B12 chromosome, but it would not distinguish between the genotypes HLA-A9,B12/A2,B12 and HLA-A9, B12/A2,BX. In surveying populations, and even families, therefore, statistical methods must often be used to estimate genotypic and haplotype frequencies. There are fairly complex methods for estimating gene frequencies (e.g. Yasuda and Kimura, 1968) as well as comparatively simpler ones (e.g. in Albert and Götze, 1977; Bender, 1979). All the methods give generally similar results.

As with other blood group, enzyme and serum group polymorphisms, HLA gene frequencies are very differently distributed in different racial and ethnic groups. Summaries may be found in Albert and Götze (1977) and Bender (1979). Among the most frequent HLA-A alleles in European Caucasians are HLA-A2, A1, A3 and A9; in African Blacks, Aw30 is frequent along with A2 and A9, while in Japanese, A9 is very frequent with A2 and A11 somewhat less so. Similar differences are seen in the alleles at other loci. The most common Caucasian haplotypes are HLA-A1,B8, A2,B12 and A3,B7.

Many of the HLA haplotypes occur much more frequently than would be expected on the basis of the gene frequencies for the alleles involved (e.g. Hiller *et al.*, 1978). In the case of two loci like HLA-A and HLA-B, linked but separable by recombination, it is expected that large numbers of recombinations between the loci will eventually bring the alleles into random association with each other, i.e., into "linkage equilibrium". The situation that is actually found in some haplotypes, where associations between certain alleles differ significantly from what would be expected on the basis of random association, is called "linkage disequilibrium". Linkage disequilibrium can be found at any pair of linked loci. Some alleles at the closely linked loci determining salivary polymorphic proteins (see in section 47) exhibit this phenomenon, for example. Details of linkage disequilibrium analysis may be found in Cavalli-Sforza and Bodmer (1971). The reasons for the disequilibria are unknown, and a number of different explanations are possible. The recombination frequency between HLA-A and HLA-B is sufficiently low (Svejgaard *et al.*, 1971; Belvedere *et al.*, 1975; Bijnen *et al.*, 1976), however, (about 0.8%) that once linkage disequilibrium is established, it will take many generations to reestablish equilibrium again.

Pious and Soderland (1977) estimated the mutational rate for the HLA-B locus, and said that their data were consistent with most of the variants having arisen by mutational events.

In 1970, Lamm *et al.* found a hint of linkage between PGM_3 and *HLA*. Somatic cell hybrid studies then enabled Jongsma *et al.* (1973) to assign PGM_3 to chromosome 6. *HLA*, PGM_3 and *SOD-B* were then shown to be syntenic by further somatic cell hybrid studies (van Someren *et al.*, 1974). Mayr *et al.* (1975) confirmed the PGM_3:*HLA* linkage. A peculiar inversion allowed the absolute assignment of *HLA* to chromosome 6 by Lamm *et al.* (1974). Thus, the human MHC, which includes the HLA loci, is on chromosome 6, and is linked to PGM_3 as well as to *GLO* and *Bf* (Weitkamp, 1976) and to a number of the polymorphic complement component loci (section 45.2). Albert and Götze (1977) said that the likely order for all the loci clustered on the short arm of chromosome 6 is PGM_3. . . . . GLO. . . . C2. .HLA-D. .Bf. .HLA-B. .HLA-C. . . HLA-A, with C4 somewhere near Bf or HLA-B.

46.3.4 The HLA-C locus

Sandberg *et al.* (1970) found an antiserum which detected an antigen that would not fit into the two locus model. A third locus, called "AJ", was postulated and was soon proven to exist (Solheim and Thorsby, 1973; Mayr *et al.*, 1973). It has also been called the third serologically defined locus, or "SD3", but is now known as *HLA-C*. The HLA-C locus is closely linked to HLA-A and -B, and crossovers between HLA-A and -C alleles and between HLA-B and -C alleles have been reported (Löw *et al.*, 1974; Hansen *et al.*, 1975; Bijnen *et al.*, 1976; Waltz and Rose, 1977).

The third locus antigens have been studied in part by a special technique called "capping" (Taylor *et al.*, 1971). A number of cell surface proteins, including HLA antigens, can, after binding to specific antibodies in the absence of complement, redistribute to form aggregates on the cell surface. They may then form a "cap" at the cell pole (capping). The aggregated surface proteins may then be taken up by the cell, or shed to the medium. Lymphocytes treated in this way show a temporary loss of the antigen involved. The finding by Bernoco *et al.* (1972) that HLA specificities can be individually capped provided a clever method for the study and characterization of these antigens.

At present, there are 8 alleles at *HLA-C* (WHO-IUIS Nomenclature Committee, 1980). They are designated HLA-Cw1 through Cw8, and the first five are considered fully established specificities. Because of the close association of the complement component polymorphic loci *C2* and *C4* with the HLA locus region, and the possibility of resulting ambiguity and confusion in the nomenclature between the alleles of *C2*, *C4* and *HLA-C*, the committee has recommended retaining the "w" designations for all

HLA-C alleles for the time being. Of the antigens defined thus far, HLA-Cw3 and Cw4 are the most frequently occurring in Europeans.

46.4 Lymphocyte Defined Antigens—A Further HLA Locus

As has been noted, the HLA-A, -B and -C locus alleles and antigens were defined by serological reactions, primarily by the lymphocytotoxicity test (discussed further below) using antibodies directed at the specificities. The antigens coded for by these alleles have thus been referred to as "serologically defined" ("SD").

Another group of antigens belonging to HLA, but detected by different methods, has also been found. These were detected by cellular reactions involving lymphocytes, and are referred to as "lymphocyte defined" ("LD"). The distinction may soon be mainly methodological, since it appears that the "LD" antigens can be detected serologically now.

When lymphocyte suspensions from unrelated individuals are mixed and incubated in culture, the cells may enlarge and transform into dividing blast cells (Hirschhorn *et al.*, 1963; Bain *et al.*, 1964). The dividing cells must replicate their DNA, and the lymphocyte stimulation reaction can thus be followed by monitoring the incorporation of radiolabelled DNA precursors (e.g. ^{3}H-thymidine). One of the two cell populations can be inactivated by treatment with radiation or mytomycin so that they cannot divide and proliferate, but can still stimulate the untreated cells in the culture to do so. This finding provided the basis for a one-way or unidirectional stimulation test (Bach and Voynow, 1966). A two-way mixed lymphocyte culture could, therefore, be analyzed as the sum of the two one-way reactions. The rationale for mixed lymphocyte culture studies was the finding (Bach and Hirschhorn, 1964) that the degree of blast transformation observed in the mixed culture was related to the degree of incompatibility for transplantation antigens, and an *in vitro* measure of transplantation incompatibility was clearly desirable. The test was developed (Bach *et al.*, 1967) into what is now called the "mixed lymphocyte culture" ("MLC"). Absence of stimulation in this procedure is interpreted to mean identity of the "MLC" antigens in the two kinds of cells. At first, MLC was thought to be just an alternative way of typing HLA antigens, because Bach and Amos (1967) observed that the lymphocytes of HLA-identical siblings did not stimulate each other in MLC, while those of HLA-nonidentical siblings did so. It was soon found, however, that the cells of unrelated HLA-identical individuals stimulated each other in MLC (Kissmeyer-Nielsen *et al.*, 1970; Schellekens and Eijsvoogel, 1970; Schellekens *et al.*, 1970; and others). These observations could be understood in two different ways: (1) that the HLA antigens were still "heterogeneous", i.e., that available typing serums did not allow for complete type determination; or (2) that the stimulation in MLC was not being brought about by HLA antigens as such, but by products of one or more "MLC" genes at a locus closely linked to the known HLA loci. Families were soon found (Amos and Yunis, 1971; Yunis and Amos, 1971; Mempel *et al.*, 1972) in whose members the HLA types and MLC behavior provided convincing evidence for a separate "MLC" locus, closely linked to HLA-B.

Recognition that the MLC tests were detecting antigens coded for by a separate locus led to efforts to devise methods for identifying different individual allelic products. MLC stimulation is a measure of difference, but not of the exact type of difference, between the antigens of the cell populations. Individuals can be selected, however, who are homozygous for certain MLC alleles. Their cells, homozygous typing cells (HTC), do not provoke stimulation in one-way MLC if the responding cells carry the same MLC allele. If a difference exists, however, a stimulation reaction is seen. The HTC technique was developed by Mempel *et al.* (1973), Dupont *et al.* (1973), Jørgensen *et al.* (1973) and van den Tweel *et al.* (1973). Another procedure for typing MLC determinants was developed by Sheehy *et al.* (1975) and Sheehy and Bach (1976), among others, and is called "primed lymphocyte typing" (PLT). This method is based on the observation that stimulated T lymphocytes kept in culture for a matter of days will give a strong secondary (or "second set") stimulation response when stimulated by cells possessing the same MLC determinant as the stimulating cells in the original culture ("priming" cells).

Following the Aarhus workshop (Histocompatibility Testing, 1975), the nomenclature committee designated the "MLC" determinant locus as "HLA-D" (WHO-IUIS Terminology Committee, 1976). The locus had previously been designated variously as "MLR-S1", "LD-1" and "MLC-1". Six specificities were provisionally recognized at that time (Dw1 through Dw6). There are presently 12 HLA-Dw specificities (WHO-IUIS Nomenclature Committee, 1980). Further information on cellular typing may be found in the reviews by Albert and Götze (1977) and Bender (1979), and in Bach *et al.* (1977), van Rood *et al.* (1977) and Bradley and Festenstein (1978).

It was found by Ceppellini *et al.* (1971) that the MLC reaction can be inhibited by certain HLA antisera, and this observation provided the beginning of a serological definition of the B-cell alloantigens. The antibodies in the HLA antisera causing the inhibition are not HLA antibodies, because they cannot be absorbed by appropriate platelets. They can be absorbed out by appropriate leucocytes, however, and eluates from these leucocytes are also active (Revillard *et al.*, 1973; van Leeuwen *et al.*, 1973). These antibodies have been better characterized by van Leeuwen *et al.* (1973), and are detecting serologically antigens on B lymphocytes that are identical or very similar to the "MLC" determinants. These antigens are also referred to as "Ia" antigens, by analogy to the findings in the mouse. Sera containing strong HLA antibodies are often found to contain strong Ia-antibodies as well (Jones *et al.*, 1975). The complex subject of human Ia antigens has been reviewed by Wernet (1976), Barnstable *et al.* (1977), Bodmer (1978b) and Winchester and Kunkel (1979). It is not yet completely clear

whether the same polymorphic antigens are being detected by the cellular (HTC and PLT) techniques, and the Ia-serological tests. The WHO-IUIS Nomenclature Committee (1978) has assigned the symbol "DR" (for "D-related") to the HLA-D antigens detected serologically. The HLA-DR designations correspond to the relevant HLA-D designation. At present, there are six fully recognized HLA-DR specificities, and an additional four provisional ones (WHO-IUIS Nomenclature Committee, 1980).

46.5 HLA Testing and Typing Procedures

Methods for HLA typing are unlike any of the methods used for the typing of blood or serum groups or polymorphic enzymes. HLA antigens are routinely typed on leucocytes, and HLA typing generally tends to be done in a relatively few specialized laboratories. Typing of HLA-A, -B and -C antigens is carried out fairly universally using some variation of the complement mediated lymphocytotoxicity test on peripheral blood lymphocytes. Special procedures which were briefly discussed in section 46.4 are used for the definition of HLA-D and -DR specificities.

The microdroplet lymphocyte cytotoxicity test introduced by Teresaki and McClelland in 1964 has become universally accepted as the method of choice for HLA antigen typing. The principle of the test lies in the fact that lymphocytes containing a particular antigen will, after reaction with specific antibody to that antigen followed by the addition of complement, exhibit a cytotoxic reaction detectable by the penetration of the cell by an added dye. Test results are evaluated microscopically. If the lymphocytes being tested lack the antigen to which the antiserum is directed, there is no cytotoxic reaction, and the added dye is not taken up by the cells. This procedure can be employed using very modest amounts ($\mu\ell$) of cells, antisera, and complement (rabbit serum is usually the complement source). The procedure has been refined and modified over the years (Mittal *et al.*, 1968; Teresaki *et al.*, 1978a). A detailed review of the developments will be found in Teresaki's Philip Levine Award lecture (Teresaki *et al.*, 1978a).

There are a number of technical problems associated with HLA typing that must be appreciated if correct results are to be obtained. These problems are discussed by Teresaki *et al.* (1978a) and by Joysey and Wolf (1978). In addition, the complexities and idiosynchrasies of HLA system antigens must be taken into consideration. Skill and experience in lymphocyte typing, and the use of carefully and thoroughly evaluated reagents, are both essential.

Efforts to standardize the lymphocytotoxicity tests used for routine HLA typing have been quite successful, although there are some variations in procedure in different laboratories (Teresaki *et al.*, 1978a; Ray, 1979). Small differences in technique can significantly affect the sensitivity of the test, however (Joysey and Wolf, 1978).

The biggest problems in HLA serology probably have to do with the antisera. Antisera must be procured, and then exhaustively evaluated in terms of specificity and cross reactivity using large panels of highly selected cells of well defined specificity. The well known cross reactivity of HLA antisera, and the fact that many serums contain a number of different antibody specificities, can cause serious difficulties of interpretation in inexperienced hands. As one example, the cross reactivity of HLA-B7 and HLA-B27 can lead to typing errors (Joysey and Wolf, 1978; Larsen, 1979). A tissue type cannot be reliably diagnosed until it has been confirmed by a battery of well authenticated antisera. Likewise, antisera must be thoroughly evaluated against large panels of cells of selected known specificity. Panels for evaluating most antisera can be smaller in number if they are highly selected (Kissmeyer-Nielsen and Kjerbye, 1972).

Since around 1965, the NIH [National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services] Serum Bank has been a major coordinating center for the exchange of HLA antisera. Catalogs of these reagents are produced periodically, and the sera are available to qualified investigators (see in Amos and Ward, 1975). Many exchanges of serums and cells take place among specialized HLA testing laboratories as well. These exchanges, and the fairly frequent international workshops, have helped considerably in the standardization of typing reagents, techniques, and nomenclature. Because of the relative scarcity of thoroughly evaluated reagents, and the complexities associated with accurate HLA typing, this work will probably continue to be restricted to a relatively small number of specialized laboratories. It may be noted that the microdroplet lymphocyte cytotoxicity test is now applicable to the determination of some HLA-D specificities as well (Teresaki *et al.*, 1978a).

46.6 Biochemical Studies on HLA Antigens

The HLA antigens are membrane-associated, and the usual problems connected with efforts to dissociate and study membrane bound proteins have faced biochemists engaged in HLA antigen characterization. Solubilization and isolation of these molecules has been carried out using a variety of techniques and extraction media, including mechanical disruption, hypotonic lysis, freezing-thawing, various salt or proteolytic enzyme (especially papain) treatments, and detergent treatments. The development of continuous lymphoid cell lines derived from normal donors has contributed to the biochemical studies by providing relatively large amounts of genetically uniform cellular material. Reviews of the biochemical work may be found in Albert and Götze (1977), Albert and Scholz (1978) and Barnstable *et al.* (1978).

There are conflicting reports in the literature about the carbohydrate content of purified HLA antigens. Some of the solubilization techniques apparently yield preparations which lack detectable carbohydrate. The consensus appears to be that there is some carbohydrate associated with the native antigens, but its exact composition and the mode of its attachment to the protein moiety have yet to be established.

Isolated HLA antigen molecules have a general structure

consisting of one heavy and one light chain. The chains are not covalently bound to one another. The heavy chain is the one coded for by the HLA locus gene, and contains the alloantigenic portion of the molecule. The MW of the heavy chain varies from 34,000 to 55,000 depending on the method used to solubilize the antigen. Strominger *et al.* (1977) presented a model of the native structure of HLA-A and -B antigens based on studies in cultured lymphocytes. The N-terminal end of the heavy chain is the one that protrudes to the outside of the cell membrane. This protruding part of the chain appears to represent a large portion of the whole molecule. A hydrophobic portion of the heavy chain was postulated to occupy the membrane itself, with a comparative short portion of the chain (the C-terminal end) protruding to the inside of the cell membrane. There are two intrachain disulfide bridges in the heavy chain. Amino acid composition studies on a number of solubilized HLA antigens from various HLA-A and -B types indicate considerable similarity. It appears that the amino acid residue at position 9 is locus-specific (Phe in the HLA-A products; Tyr in the HLA-B ones), but the nature of the differences giving rise to the alloantigenic specificities have not yet been clarified. Most of the data suggests that the carbohydrate moiety is not involved in determining antigenic structure or specificity.

The light chain (MW 11,600) of the HLA-A and -B molecules is associated with the N-terminal end of the heavy chain, according to the structure proposed by Strominger *et al.* (1977). The light chain is apparently not involved in determining alloantigenic specificity, and the light chain has been identified as β_2-microglobulin. β_2 microglobulin is found in small amounts in various body fluids and was first isolated and purified by Berggård and Bearn (1968). It has 100 amino acid residues and a single disulfide bridge formed by Cys residues at positions 25 and 81. There is no carbohydrate in the purified preparation. There is some sequence homology of β_2-microglobulin with the constant region of the IgG heavy chain (Smithies and Poulik, 1972). β_2-microglobulin synthesis is controlled by a gene on chromosome 15 (Goodfellow *et al.*, 1975; Zeuthen *et al.*, 1977), and its structural gene is completely separate from the HLA complex locus, therefore.

Although there are fewer detailed studies on the HLA-C antigens than on the -A or -B molecules, the data indicate that at least the general structure is very similar.

Berg (1971b) reported the curious observation that there are amino acid compositional similarities between HLA antigens, the Lp(a) protein and the LDL of serum (section 45.1). Charlton and Zmijewski (1970) found that HLA-A7 people had a soluble HLA-A7 in their plasma which occurred as a low density lipoprotein. Orr *et al.* (1979) recently noted that an 88 amino acid residue fragment of the HLA-B7 heavy chain showed significant structural homology with the constant domains of immunoglobulins.

The structure of the HLA-DR antigens (Ia-antigens) is somewhat different from that of the HLA-A, -B and -C molecules. The data thus far indicate that the DR antigens resemble mouse and guinea pig Ia antigens. Snary *et al.* (1977) have presented a model for the structure of these molecules with which the data of Klareskog *et al.* (1977) appear to be consistent. The molecule is a glycoprotein consisting of two polypeptide chains of MW about 33,000 and 28,000. The chains are not covalently linked, and both of them have associated carbohydrate. The 33,000 peptide is suggested to be the one coded for by the HLA-DR genes, and accordingly, the one containing the allotypic site. Synthesis of the 28,000 peptide might well be controlled at a completely different locus.

46.7 Medicolegal Applications

46.7.1 Disputed parentage

The use of the HLA polymorphism in disputed parentage represents its only significant medicolegal application at the present time. The extraordinary extent of the polymorphism and its widespread distribution in populations make HLA the most singularly powerful system for parentage investigations. This fact may be appreciated by examining Table 18.1. The probability of exclusion of a falsely accused father using HLA typing is very high, and values ranging from about 80% to over 90% are quoted by various authors. The PE is dependent, of course, on how many specificities can be typed, and on the distribution of haplotypes in the population in question. The system is so powerful in making distinctions in the population because of the large number of haplotypes and their widespread distribution in the population. With 17 alleles at HLA-A and 28 at HLA-B, Bender (1979) said that 17 homozygous and 136 heterozygous A types, and 28 homozygous and 378 heterozygous B types were possible. Since the antigens are inherited as haplotypes, it must be considered that there are then 476 different A,B haplotypes which allow for about 113,500 combinations! The most frequent phenotype occurs in only about one of 100 persons. Bender (1979) also said that at least two-thirds of the German population have two different A and two different B alleles (a "full house" in laboratory jargon).

Application of HLA typing to parentage cases has been practiced for some years, and application of HLA to paternity cases is discussed by Mayr (1971 and 1974), Soulier *et al.* (1974), Spielmann and Seidel (1974), Spielmann *et al.* (1974), Speiser (1975) and Miller, in AABB (1978). Calculations of PE were discussed by Mayr and Pausch (1975), and the principles underlying calculation of the probability of paternity where no exclusion is obtained were given by Mayr (1972). Hummel and Conradt (1978) noted that very low probability of paternity values could be obtained in some cases, even when the PE value for exclusion of a falsely accused father was high, and they tended to take the view that the probability of paternity calculation was the only meaningful one in a given case.

In this country, Dr. Teresaki has been a leader in the application of HLA typing in paternity cases (Teresaki, 1978; Teresaki *et al.*, 1978b). If ABO and Hp are typed in addition

to HLA, probabilities of paternity are at least 95% in the non-exclusion cases. In a number of states, blood grouping evidence is not admissable unless it is exculpatory (i.e. results in excluding the putative father), but this situation is changing somewhat as members of the legal community become more aware of the present status of blood testing (e.g. Seider, 1980). Lee (1975) surveyed 24 countries, and found that HLA typing was used in paternity cases in just under half of them. In this country, it is one of the seven systems recommended by the joint AMA–ABA Committee (Joint AMA–ABA Guidelines, 1976), along with six blood group systems (ABO, Rh, MNSs, Kell, Duffy and Kidd). For some reason, this committee chose to overlook isoenzyme and serum group systems altogether. Polesky and Krause (1977) found in their survey of American laboratories that about 17% of AABB referene laboratories could do HLA testing, but less than 2% of other laboratories surveyed had this capability.

The applicability of HLA typing in some uncommon situations has been described. Speiser *et al.* (1974) discussed the situation in which the putative father could not be tested. Teresaki (1978) had a case in which the mother could not be tested. In both situations, exclusions could be obtained. Two very rare cases of so-called superfecundation have recently been diagnosed by HLA typing. In both, fraternal twins were shown to have different biological fathers (Teresaki *et al.*, 1978c; Bertrams and Preuss, 1980).

46.7.2 HLA Antigen typing in dried blood

Only a few studies have been carried out on bloodstain typing. Rittner and Waiyawuth (1974) obtained a high percentage of correct results using an inhibition of lymphocytotoxicity test. They then (1975) compared microlymphocytotoxicity and microcomplement fixation procedures on bloodstain material, and concluded that the latter would be better suited. A number of specificities could be reliably detected, but relatively large amounts of dried blood were used in the tests. Newall (1979) evaluated two different versions of microlymphocytotoxicity inhibition for the typing of HLA-A2 and B5 in dried bloodstains of varying age. The two-stage extraction-absorption test was not found to be very satisfactory. Better, more reliable results were seen with a one-stage procedure. Antisera varied in their ability to give unequivocal typing results, and stains from different persons that contained the same antigen gave differing degrees of inhibition, especially with older stains. Stains up to 10 weeks old could be typed correctly, however, if three different antisera were used in each test. Hodge *et al.* (1979 and 1980) recently investigated HLA-A1 typing in 4 cm long bloodstained threads using inhibition of microlymphocytotoxicity procedures. Three antisera were used in parallel. Most stains could be typed correctly even in stains over 100 days old. Still, a few false positive and negative typings were observed in the samples. The well known HLA-A1-A11 cross reaction could be seen in HLA-A2,A11 stains with one of the anti-HLA-A1 sera known to be cross reactive with HLA-A11. While these initial results are encouraging, more groundwork remains to be done before this powerful polymorphic system can be exploited in bloodstain typing.

Hillman and Shaler (1981) have recently explored procedures for the recovery of leucocytes from dried bloodstains. Using special extraction techniques, relatively high recoveries are possible. The leucocytes thus isolated from bloodstains might enable HLA typing to be carried out much more readily than is possible with whole bloodstains. Such leucocytes can be used for cytologic sexing studies (section 48), and for the determination of genetic markers that are expressed in white cells but not in red cells.

It may eventually be of medicolegal interest that HLA antigens appear to be present in semen (Singal *et al.*, 1971; Singal and Berry, 1972) and on sperm cells (Halim and Festenstein, 1975; Festenstein *et al.*, 1977).

SECTION 47. POLYMORPHIC PROTEINS OF HUMAN SALIVA

47.1 Introduction

Saliva contains a variety of proteins and enzymes, many of which it shares with other body fluids and tissues. Some of the antigens, enzyme and serum group system proteins that exhibit genetic polymorphism and occur in saliva as well as in other tissues and fluids have been discussed elsewhere in this book. Soluble ABH and Lewis blood group substances (sections 19.8.1, 19.8.3.1 and 20.4) are found in saliva as well as in many other body fluids and tissues. Likewise, the antigen Sd^a (25.8) can be found in saliva. Some immunological studies which included identification of some of the proteins of saliva were discussed in section 11.4. Saliva contains a few proteins which appear to exhibit inherited variation (presence or absence) that have been detected by their reactions with various lectins. One of these, which was called "L", was detected by its reaction with a lectin from *Lotus tetragonolobus* (section 19.8.3.1).

In 1964, Niswander *et al.* detected a component in saliva by immunoelectrophoresis and reaction with a rabbit antiserum raised against human parotid saliva. It was called "SC_1" (for "salivary component" migrating most anodally by immunoelectrophoresis at pH 8.6). The component migrated in the "albumin" position, but was not albumin, nor was it identical to or associated with $ABHLe^a$. It did not occur in serum. It was inherited, but appeared to be subject to post-translational modification, and the inheritance pattern was not fully clear. The relationship of SC_1 to the proteins discussed in section 47.2 is not clear. Two more saliva antigens were detected by Balding and Gold (1973). They were studying bacterial hemagglutinins from *Clostridium botulinum* types C and D. It was found that some but not all salivas from different people would inhibit these reactions. The antigen inhibiting the type C agglutinin was called CbC, and that inhibiting type D activity was called CbD. The characteristic was inherited, and the responsible genes, called Sal^{CbC} and Sal^{CbD}, respectively, had frequencies of about 0.73 and 0.27 in a Caucasian population. In 1975, Wiener and Moon described an antigen called "C1" on red cells and in saliva. The antigen was defined by a lectin prepared by absorbing extracts of the seeds of *Clerodendron trichotomum* Thumberg (from Korea) with selected group O red cells, and was said to be associated with the ABHLe macromolecule.

Saliva contains a number of enzymes, the most important of which for forensic purposes is probably amylase (at least at the present time). The use of amylase as an identification marker was discussed in section 11.3. The polymorphism of the salivary amylase (AMY_1), as well as that of pancreatic amylase (AMY_2) was discussed in section 37.3. The use of salivary alkaline phosphatase as an identification marker was covered in section 11.2. Pini Prato (1970) noted two alkaline phosphatases in human saliva following polyacrylamide gel electrophoresis. The relationship of these isozymes to the other human alkaline phosphatases (section 37.2) is not yet clear. Detection of the PGM_1 isozymes in the cellular fractions of saliva has been reported (section 27.4.3), although PGM should probably not be regarded as a salivary enzyme as such. The immunoglobulin markers of the Gm and Km system can be detected in saliva, and this matter was covered in section 44.6.3.

Several systems of salivary proteins and enzymes, which have not yet been discussed, exhibit genetically controlled variation by electrophoresis or isoelectric focusing. The rest of section 47 is devoted to a discussion of these systems. Most of the work is comparatively recent, and only a handful of laboratories appear to be engaged in these studies. The most comprehensive review of this material was given by Azen (1978). I am indebted to his review in helping to organize some of the material in this section. A useful and comprehensive review of methods employed in studying salivary polymorphisms will be found in Tan and Teng (1979).

47.2 Polymorphic Salivary Protein Systems

47.2.1 Acidic protein (Pa system)

Polymorphism in an acidic protein in saliva (as against the basic ones of the Pb system discussed below) was described by Friedman *et al.* (1972), and studied further by Friedman *et al.* (1975). The Pa protein, which is either present [Pa(+)] or absent [Pa(−)] was detected using acid urea starch gel electrophoresis, and a stain designed to detect argninine-rich proteins. Other electrophoretic systems were used to study Pa further, and to compare it with other salivary proteins. Pa can be typed in submandibular or parotid saliva, but something like half the whole saliva samples were found to be unsuitable for typing, even if frozen immediately after collection in preparation for lyophilization. Saliva preparations are usually frozen, and lyophilized, and the lyophilizate later reconstituted for electrophoretic study (Friedman *et al.*, 1975). The locus controlling the Pa protein was designated *Pa*, and the protein was clearly inherited as an autosomal dominant as indicated by studies on a number of families. Friedman *et al.* (1975) call the phenotypes Pa(+) and Pa(−) and the genes Pa^+ and Pa^-. Azen (1978) calls the phenotypes Pa 1 and Pa 0, and the genes Pa^1 and Pa^0, respectively (because there is now a "Pa^2"). Pa(−) × Pa(−) matings did not give rise to any Pa(+) children. Gene frequencies for Pa^- (Pa^1) were approximately 0.21, 0.14 and 0.42 for American Whites,

American Blacks and Orientals, respectively. In 1977, Azen described a rare variant of Pa, called Pa 2. Pa 2 is a separate protein which is seen on the typing gels, and was accounted for by a rare allele Pa^2. The finding of the Pa^2 accounts for the change in nomenclature noted above.

Friedman and Merritt (1975) partially purified the Pa (Pa 1) protein, and found that it was rich in Pro, Glu and Gly, low in Thr, and that it had no Tyr or Cys. The pI was 3.9–4.5. Azen and Denniston (1974) and Azen (1977) found, however, that Pa 1 was a disulfide-bonded dimer, using disulfide bond splitting agents and ^{14}C-iodoacetamide to identify the products. Friedman and Merritt (1975) mentioned that there were some associated acidic proteins in saliva, called "Pa II", to distinguish them from "Pa I", the polymorphic Pa.

47.2.2. The Pr and Db systems and their relation to Pa

The Pr system polymorphism was originally described by Azen and Oppenheim (1973). The proteins exhibiting this polymorphism had previously been characterized to some extent by Oppenheim *et al.* (1971). Four proteins made up the system, and were detected by alkaline slab polyacrylamide gel electrophoresis using parotid saliva which had been concentrated 5–10 fold by lyophilization and reconstitution in gel buffer. The proteins are visualized by means of acidified 3,3′-dimethoxybenzidine. The proteins were negatively stained, i.e., the background changed color while the protein zones did not. More contrast could be obtained if a second staining step was done with a peroxide solution. Apparently, some of the DMB precipitated in the gel in the first step, everywhere except at the protein zones, and could then react with the peroxide in the second step to give a brown background. The basis for this unusual staining reaction is not known (Azen and Oppenheim, 1973; Azen, 1978). Three patterns could be distinguished, and family studies showed that these could be accounted for by two alleles, called Pr^1 and Pr^2. The Pr^1 gene was responsible for the protein bands called Pr 1 and 3 (originally I and III), while Pr^2 was responsible for Pr 2 and 4 (originally II an IV). Frequencies of the Pr^1 allele were about 0.73 in Whites, 0.8 in Blacks and 0.84 in Chinese.

The Pr proteins are especially rich in Gly, Glu and Pro and have pI in the range of 4.09–4.71. Pr 1 has a MW of 12,300 and Pr 3 of 6,100 by sedimentation ultracentrifugation (Oppenheim *et al.*, 1971). Immunological evidence suggests that the Pr proteins separable by isoelectric focusing are very similar if not identical in antigenic structure (Friedman and Karn, 1977).

Azen and Denniston (1974) studied the Pr proteins more closely, and found that two additional proteins at the so-called "X" position (slightly slower than but close to Pr 1) could be distinguished by changing the pH or gel concentration for electrophoresis. One of these proteins had a disulfide bond, and is now known to be identical to Pa 1 (Azen, 1977 and 1978). Some people have the "X" proteins while others do not. One of the X proteins is usually found associated with Pr^2 products, and only rarely with Pr^1 products. The other X protein is always associated with a product of Pr^1. These findings led Azen and Denniston (1974) to suggest that there were at least two additional alleles at the *Pr* locus, called $Pr^{1'}$ and $Pr^{2'}$. The data also suggested that the gene locus responsible for the Pa 1 protein, *Pa*, and the *Pr* locus genes have undergone occasional recombination. In addition, all Pr 2′ individuals are Pa 1, and most Pr 1′ people are Pa 0, but an occasional Pr 1′ individual is Pa 1. This suggests a significant linkage disequilibrium between *Pr* and *Pa*. There is now no doubt about the close linkage between the two loci (Yu *et al.*, 1978).

Azen and Denniston (1974) found another pair of polymorphic proteins, called "Db" (for "double band"). These, like Pr, were detected by negative staining with dimethoxybenzidine after electrophoresis on polyacrylamide gels. The double bands, one of which is between Pr 2 and Pr 3 and the other of which runs behind (cathodal to) "X", are either present or absent. The phenotypes are denoted Db+ and Db−. The locus controlling the variation is called *Db*, and the gene determining Db+ is dominant. Approximately 12% of Whites, 56% of Blacks and 7% of Chinese were Db+. *Db* is very closely linked to *Pr* (Azen and Denniston, 1974; Friedman *et al.*, 1975; Azen, 1977).

Extensive studies on the linkage relations of *Pa*, *Pr* and *Db* have been carried out by Azen and Denniston (1974), Friedman *et al.* (1975), and Yu *et al.* (1978 and 1980a). The evidence suggests that there are three separate, but closely linked, loci, *Pr* and *Pa* being closer than *Pr* and *Db*. Definite recombinations have been seen in the family material of Yu *et al.* (1978), thereby proving the existence of separate loci. Azen (1978) said that the *Pr* locus had three definite alleles, Pr^1, $Pr^{1'}$ and Pr^2, that the *Pa* locus had Pa^0, Pa^1 and Pa^2, and that *Db* had Db^+ and Db^-. A reasonable (but not yet proven) order for the loci is *Pa. . .Pr. . .Db* (Yu *et al.*, 1980a).

47.2.3 Basic protein (Pb)

Polymorphism in basic proteins of parotid saliva was detected by Azen in 1972, using acid urea starch gel electrophoresis and staining for argining-rich proteins. Three patterns are seen, and called 1, 2-1 and 2. The proteins show asymmetric patterns in the homozygous types, and there is variation in band intensity in different samples of the same type. Pb 1 homozygotes show four bands, called "a", "b", "d" and "e", while Pb 2 homozygotes show only a "c" band. The letters represent the order of the bands on the plate. The patterns can be explained by a pair of codominant alleles, Pb^1 and Pb^2, at an autosomal locus (Azen, 1973). Pb^1 frequency is about 0.84 in Blacks, but over 0.99 in Whites; the Pb system is thus polymorphic principally among Blacks.

The Pb proteins have been purified and partially characterized (Peters and Azen, 1977). The MW ranged from 5,800 to 7,200, and the proteins are extremely basic (pI > 9.5). Approximately 45% of the amino acid residues are the basic His, Lys and Arg ones. The proteins contained no carbohydrate, and lacked Cys, Pro, Thr, Val, Met and Try.

There is evidence from the biochemical and amino acid composition and sequence studies (Peters and Azen, 1977) that the polymorphism is due to a combination of allelic differences and post-translational modifications involving proteolysis and deamidation. There is probably a single amino acid difference (Glu vs Gln) in the primary gene products. This model helps to explain the peculiar asymmetric banding patterns in the homozygous types. Peters *et al.* (1977) found a larger MW protein in parotid saliva, called "post-Pb protein", which most closely resembled the Pb "e" band protein in MW. It was immunologically cross-reactive with an antiserum raised against the Pb proteins, and was first thought to be a possible precursor of the Pb proteins. It is now believed that this is not the case, however (Azen, 1978), and this protein is probably determined at a different genetic locus.

Despite a considerable amount of effort by the Wisconsin group, the Pb proteins have not been assigned a physiological function thus far.

47.2.4 Other polymorphic salivary proteins

47.2.4.1 The major parotid salivary glycoprotein (Gl). In addition to the acidic proline-rich proteins which have been shown to exhibit genetic polymorphism (Pa, Pr and Db systems—see above), parotid saliva contains various basic proline-rich proteins. Unlike their acidic counterparts, many of them are glycosylated. Earlier studies (e.g. Friedman *et al.*, 1971) have shown that they are heterogeneous, differing in size, charge and MW, and there was speculation that this variability might have a coherent genetic basis. In 1979, Azen *et al.* carried out extensive studies on the major glycosylated protein in parotid saliva, and showed that it exhibits discrete, genetically controlled molecular variants.

Parotid saliva was extensively studied using a polyacrylamide gel electrophoretic system and acidic (pH 2.4) buffers. Fairly high gel concentrations were required (routinely 9% and sometimes 13.5%). Thick (6 mm) gels were employed, and sliced prior to staining for carbohydrate. The optimal resolution of the bands was obtained with samples that had been subjected to neuraminidase treatment. Other staining overlays served to distinguish the proteins of the Pa, Pr and Db systems from those under study here.

Eleven phenotypes of the major glycosylated proteins were observed, five of which appeared to represent homozygosity at the controlling locus. The system was termed "Gl", and the homozygous phenotypes Gl 1, 2, 3, 4 and 0. In addition, heterozygous phenotypes 1-2, 1-3, 1-4, 2-3, 2-4, and 3-4 were seen. There are four band positions in the system used, and they represent (in order from origin to cathode, in the direction of the run) the 4, 1, 2, and 3 bands. Gl 0 has no bands. Five codominant alleles at an autosomal locus account for the phenotypes, and they were called Gl^1, Gl^2, Gl^3, Gl^4 and Gl^5. Gene frequences seen in 143 Whites were $Gl^1 = 0.742$, $Gl^2 = 0.04$, $Gl^3 = 0.155$, $Gl^4 = 0.017$ and $Gl^5 = 0.046$, while in 82 Blacks they were $Gl^1 = 0.459$, $Gl^2 = 0.05$, $Gl^3 = 0.337$, $Gl^4 = 0.044$ and $Gl^5 = 0.11$. Homozygous types could not be distinguished from the heterozygous condition in which one of the alleles was Gl^5 (e.g. Gl^1Gl^1 and Gl^1Gl^5 people type as Gl 1, and so forth). Studies on 41 families with 146 children were completely consistent with the postulated mode of inheritance. Studies on informative families strongly suggested linkage of *Gl* with *Pr* and *Db*, thus placing it in the *Pa-Pr-Db* region. Products of *Gl* show evidence of strong linkage disequilibrium with those of *Pr*, *Db* and *Pa*. Biochemical studies on the Gl 1 and Gl 4 proteins indicated similarity in amino acid composition, and similarity to the composition of Pr 1. The polymorphic forms of Gl proteins showed microheterogeneity due to differences in size and charge, and the polymorphism seemed to be accounted for on the basis of MW differences in primary gene products.

47.2.4.2 Pm. In 1977, Ikemoto *et al.* described another polymorphism in parotid saliva using a modification of the acid urea starch gel electrophoretic system described by Azen (1972 and 1973). The Pm protein migrates in between Pa and Pb proteins (the designation "Pm" stood for "salivary parotid middle band protein"). The Pm protein is either present or absent in a given individual, and its presence or absence can be determined in whole saliva, suggesting that it is one of the more stable of the salivary parotid proteins. Family studies indicated that the protein is simply inherited as a Mendelian dominant, and the *Pm* locus is autosomal. *Pm* is apparently not linked to *Pa* or *Pb*. In a sample of 195 Japanese, the Pm^+ frequency was about 0.38. 20 Chinese were also typed, and although the number is small, Pm^+ was about 0.3 by my calculations. Azen and Denniston, in a personal communication to Yu *et al.* (1980b), have found that *Pm* is closely linked to *Pr* and *Db*.

47.2.4.3 Ph. In 1979, Ikemoto *et al.* reported on the polymorphism of a further protein in parotid saliva, detected by SDS-polyacrylamide gel electrophoresis (Ikemoto *et al.*, 1979a). This protein differed from Pb, Pa, Pr, Db and Pm, and was designated "Ph", for "salivary parotid heavy protein", because it appears to have a larger MW than the other parotid proteins (this observation had been previously reported by these authors in the Japanese literature). The MW of the Ph protein was determined to lie between 68,000 and 155,000 by SDS-polyacrylamide gel electrophoresis. Ph, like Pm, is either present or absent, and the Ph+ condition is simply inherited as a Mendelian dominant at an autosomal locus. Among 218 Japanese subjects, Ph^+ frequency was about 0.026 with Ph^- about 0.974. *Ph* was not associated with *Pr*, *Db* or *Pm*, but was associated with *Pa*. Ph was a glycoprotein, and the authors said that its relationship to Gl would require further study.

47.2.4.4 Sal I and Sal II. In 1974, Balakrishnan and Ashton described two polymorphic proteins in parotid saliva, detected by polyacrylamide gel electrophoresis at pH 8. They said that this work had been reported at the 4th International Congress of Human Genetics in Paris, and that these proteins did not correspond to the Pb proteins of Azen (1972 and 1973). The polymorphism was detectable in whole or in parotid saliva. The complex pattern of bands

could be divided into six regions, and the variation was observed in region 4. Two bands were involved; both could be present or absent. In addition, either one could be present in the absence of the other one. The proteins were inherited, and the most viable genetic explanation postulated two loci called *Sal I* and *Sal II*, each of which had a dominant and a recessive allele. *Sal I* controlled the "fast" band, and its alleles were designated *F* and *f*; the alleles of *Sal II* were correspondingly designated *S* and *s*. The "null" condition (no bands) was attributed to the *ffss* genotype. Azen (1978) said that examination of the patterns in the photograph of Balakrishnan and Ashton (1974) suggested that the two bands might represent two of the Pr proteins. However, the genetic analysis, and particularly the finding of the "null" phenotype, would not fit with this explanation.

47.3 Polymorphic Salivary Enzyme Systems

The best studies of polymorphic enzymes in saliva is probably amylase, and this system has been discussed elsewhere, as noted in section 47.1. In this section are discussed salivary acid phosphatases (Sap), esterases (Set or Set_1), Glc-6-phosphate dehydrogenase (Sgd) and peroxidase (SAPX).

The presence of an acid phosphatase activity in saliva was noted by Giri in 1936. It had a higher activity with hexose diphosphate than with glycerophosphate, and the pH optimum was 2 to 3 units higher with the former. Using polyacrylamide gel electrophoresis, Tan and Ashton (1976a) found five patterns of salivary acid phosphatase, and one deficient type. These patterns could be explained on the basis of two separate genetic loci, called *Sap-A* and *Sap-B*. *Sap-A* had three alleles, $Sap\text{-}A^A$, $Sap\text{-}A^{A'}$ and $Sap\text{-}A^0$, while *Sap-B* had two alleles, $Sap\text{-}B^B$ and $Sap\text{-}B^0$. Operation of these loci gave rise to the phenotypes AB (which was common), B, AA', A'B, AA'B, A and O. The relationship of these isozymes to other human acid phosphatases (section 29.3) will eventually want some further explanation.

In 1976, Tan found polymorphism among a pair of carboxylesterase bands in saliva using polyacrylamide gel electrophoresis and detection with α-naphthyl acetate and Fast Blue RR salt. Of the two bands, a fast one and a slow one, both or either could be present in a given person, giving the phenotypes F, FS and S. The locus controlling this polymorphism was called *Set-1*, and the observations could be understood in terms of two alleles, $Set\text{-}1^F$ and $Set\text{-}1^S$. $Set\text{-}1^F$ had a frequency of about 0.61 among Caucasians and about 0.5 among Japanese, both in Hawaii. As with the case of Sap isoenzymes noted just above, the relationship of Set-1 isozymes to the other carboxylesterases of human tissues and their genetic loci (section 31.3.6) will require additional study.

Tan and Ashton (1976b) described a fairly well distributed polymorphism in salivary glucose-6-phosphate dehydrogenase, denoted "Sgd" (it may be recalled that the locus denoting the red cell Glc-6-PD is often denoted *Gd*—see in secton 33.1). Polyacrylamide gel electrophoresis was used to separate the isoenzymes, and three phenotypes called 1, 2-1 and 2, were observed. The best genetic explanation for the data was an autosomal locus *Sgd*, with a pair of codominant alleles Sgd^1 and Sgd^2. The fact that *Sgd* is autosomal shows that it is separate and distinct from *Gd*, which is well established as being X-linked. The Sgd^1 frequency was about 0.75 in Caucasians, 0.66 in Japanese and 0.7 in Chinese, all living in Hawaii.

Genetic polymorphism in salivary (parotid) peroxidase (SAPX) was described in detail by Azen (1977). The isoenzymes were detected in concentrated parotid saliva samples by polyacrylamide gel electrophoresis in Tris-lactate buffers at pH 2.4. The gels were then washed in alkaline buffers to bring the pH to the 8.0-8.4 range prior to detection of activity. Detection was carried out using a modification of the procedure described by Uriel (1958) for Cp detection (section 45.5). It may be recalled that Cp is often detected by taking advantage of its oxidase activity. Azen (1977) found two comparatively common and one rare SAPX phenotypes. SAPX 1, the most common type, had a major fast ("F") band, a faster minor "f" band (probably a conversion product of F), and several slower bands. SAPX 2, the next most common type, showed a major slow ("S") band, and several associated slower bands, the first of which ("s") probably represents a conversion product of S. SAPX 3 was seen in one family. These phenotypes could be accounted for on the basis of three alleles, $SAPX^1$, $SAPX^2$ and $SAPX^3$. One informative family in the study showed that there was an intimate relationship between SAPX and Pa. This finding implied that SAPX would also show a relationship with Pr and Db as well, and this suspicion was confirmed. Extensive biochemical studies on the proteins and genetic studies on the interrelationships of the determining genetic loci showed that there is a perfect correlation between *SAPX* and *Pa* products. $SAPX^1$ behaves as a recessive, while $SAPX^2$ and $SAPX^3$ behave as complete dominants. The SAPX 2 and 3 products appear to be genetically controlled modifications of SAPX 1, rather than different primary gene products. Pa 1 is always associated with SAPX 2, and Pa 2 is always associated with SAPX 3. The *Pa* locus may be regarded, on the basis of these data, as a kind of "modifier" locus, whose products modify the SAPX 1 type. When the Pa type is Pa O, the SAPX type is 1. It is probable that Pa 1 or Pa 2 monomers complex with SAPX 1 through disulfide bonds to yield SAPX 2 or SAPX 3.

47.4 Typing Salivary Polymorphic Proteins

Polyacrylamide gel electrophoretic methods for typing the salivary proteins and enzymes were collected and organized by Tan and Teng (1979) in a useful summary paper. Typing some of the proteins in a reproducible way is obviously somewhat involved (Azen, 1978). More than one electrophoretic system must be employed to discern all the variants in some systems. Azen (1978) gave a thorough discussion of the most optimal typing methods for the various systems, based upon his considerable experience.

Friedman and Allushuski (1975) described a special electrophoretic system for defining Pr-Db-Pa types. It appears that the typing of the polymorphic isoenzymes, leaving aside SAPX which is closely associated with Pa, may be more straightforward.

Most workers have used parotid saliva for this work, at least in the case of the nonenzymatic proteins. This material is collected in a special way in a little cup, quickly frozen, and then lyophilized. Reconstitution of the lyophilizate for typing results in a several-fold concentration of the proteins. Many authors have noted that these proteins seem to be especially subject to proteolytic degradation, which changes the electrophoretic patterns and results in the inability to determine the type. Whole saliva is considered unsuitable for typing many of the proteins, apparently because of the rapid proteolytic activity that occurs in the oral cavity. The apparent sensitivity of the proteins to degradative change, and the necessity to concentrate even fresh material in order to diagnose the types in several of the systems, indicate that the nonenzymatic proteins may not be very suitable candidates for saliva stain identification work. There is no reason why specialized laboratories could not employ some of the systems, which have simple and clear cut inheritance patterns, in disputed parentage cases. Indeed, some of the systems are beginning to find their way into medicolegal work already (see immediately below).

47.5 Medicolegal Applications

With a single exception, to be noted below, the few papers on medicolegal applications of the salivary polymorphic proteins have been concerned with disputed parentage. Ikemoto *et al.* (1979b) carried out extensive population studies on the Pa, Pr, Db, Pm, Ph and Amy_1 systems in Japanese. The data were compared with published data for other populations from various sources. The probability of excluding a falsely accused father using each of the systems, and for the Pa, Pr, Db, Pm and Ph systems combined, was calculated from the Japanese population data. They noted that the combination of bloods groups, isoenzymes, and serum group systems currently employed for paternity testing (which did not include HLA) had a combined PE of about 0.92. The five salivary systems gave a combined PE of 0.305, and when combined with all the other marker systems, raised the overall CPE to 0.944. The value calculated for the combined PE (CPE) of the five salivary systems is, however, almost certainly in error on the high side. The Japanese workers assumed independent segregation for all the loci in doing the computation, and it is now clear, as Yu *et al.* (1980b) pointed out and as was discussed above, that Pa, Pr, Db and Pm are all closely linked, and that some allelic combinations at these loci show significant linkage disequilibria. Accordingly, independence of the loci cannot be correctly assumed in computing the PE for any combinations of these systems.

Pronk *et al.* (1979) briefly noted some of the problems associated with changes in the Pb patterns in saliva between the time of collection and that of actual analysis. These alterations are of a proteolytic nature.

Hayashi and Hayashi (1979) have described a polyacrylamide disc gel electrophoretic procedure for Set phenotyping, which was applicable to whole saliva and to saliva stains up to 6 weeks old. The conditions used to examine the stains were slightly different from those used for fresh material (which was frozen at once after collection, and thawed just prior to typing). The polymorphism was recommended for paternity investigations and as an identification marker in salivary stains.

SECTION 48. POLYMORPHIC PROTEINS IN HAIR

A number of genetic markers have been detected in hair, which shares them in common with blood and other tissues. These markers have been discussed in previous sections devoted to the various systems.

ABO grouping of hair was discussed in 19.10.7.1. ABO is the only blood group antigen marker system that has been detected in hair, and the only marker that is present in the hair shaft. There seems to be no doubt that the glycoproteins which possess ABH antigens are present in the hair shaft. Yet, while some workers have reported entirely reliable results in typing hairs, others have found that the type corresponding to that of the blood cannot always be determined correctly.

A number of isoenzyme markers which are expressed in red cells as well as in tissues can be diagnosed in the cells of the hair root sheath. These markers appear to be determinable about as reliably in hair roots as they are in blood (though, perhaps, many are determinable longer in dried blood than in plucked hairs). Among these are PGM_1 types and subtypes (27.4.3), ESD (31.4.4), PGD (33.2.4) and GLO (34.5.2). PGD aside, these systems give reasonably good discrimination in most populations, and would yield considerable individualizing information if they were all used on the same sample. A root sheath is required, however, in order to make typing possible.

Hair is common physical evidence, and techniques other than morphological comparisons that might help to individualize human hair would be most welcome in the forensic science laboratory. Many hairs encountered in case work lack root sheaths, so the enzyme markers cannot be used. And, since everyone is not in agreement about the reliability of ABO typing, morphological comparison of questioned and known hairs is usually the only remaining approach. While such comparisons have indisputable value, especially in excluding common origin, less subjective procedures would be desirable.

Dr. Baden and his collaborators at Harvard have recently carried out extensive studies on the structural proteins of hair. The data indicate that there are detectable differences in these proteins in hairs from different individuals, and that some of these differences are simply inherited. The major structural proteins of hair (keratins) consist of fibrous proteins and the amorphous matrix proteins. The fibrous proteins consist of a number of different polypeptides, having many CySSCy residues and MW in the range of 40,000–58,000. These proteins can be separated and examined by disc polyacrylamide gel electrophoresis if they are first reductively denatured in urea, and the S-carboxymethyl derivatives prepared. Baden *et al.* (1975) reported a genetic variant in one of the peptides of the fibrous proteins using this technique. The variant type occurred in about 5% of Caucasians. Hairs from any body site in variant persons showed the variant pattern. Four families were studied, and indicated that the trait was autosomal and dominant. Interestingly, the characteristic variant pattern could also be detected in nails.

The matrix proteins are a little more difficult to separate, but Lee *et al.* (1978) reported a procedure for doing so. In about 300 people, two variant proteins were detected that showed as extra bands in the usual pattern. The extra variant bands were called V_1 and V_2. V_1 was inherited in one mother-child pair. The inheritance of V_2 was not studied. Another variation was seen in which one of the usual bands was significantly reduced. This pattern was reproducible in people who exhibited it, and it appeared to have been inherited in one family. These data were regarded as preliminary, suggesting further family and population studies as well as searches for additional variation. While this work is at an early stage, the results do seem to offer hope that detectable polymorphic protein systems may well be present in the structural proteins of hair. Further study will be needed before these systems can be exploited for medicolegal work.

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