UNIT VI.
ISOENZYMES

SECTION 26. INTRODUCTION TO ISOENZYMES

The isoenzymes are an important class of genetic markers in human blood and body fluids, and a number of them have come to be applied quite widely in medicolegal investigations in the past twenty years or so. The existence of multiple molecular forms of enzymes as a general and significant biological phenomenon was recognized about 25 years ago. A very large number of enzymes from many species occur in multiple molecular forms. Those in the human species which are under the control of polymorphic genetic loci have potential applicability to medicolegal problems.

In 1957, Hunter and Markert reported that zone electrophoresis on starch gels, perfected by Smithies in 1955 (section 2.3.4) for serum protein separations, was applicable to the separation of enzymes from crude extracts if histochemical localization techniques were used to locate the zones of activity. They found the technique applicable to esterases, tyrosinases and phosphatases, and said that it was probably applicable to any enzyme system which could be resolved on starch gels, and for which a suitable histochemical localization procedure could be devised. The "activity stained" gel was referred to as a "zymogram". This relatively simple technique was quickly adapted to the study of many different enzymes from many different sources.

There were indications prior to the 1950's that enzymes might exist in more than one form, but it was not clear whether the observed heterogeneity was real or apparent. Vesell and Bearn (1957 and 1958), for example, observed heterogeneity of lactate and malate dehydrogenase activities in human serum upon electrophoretic separation of the serum proteins, and the activity patterns could be different in certain disease states. That the heterogeneity was indeed a property of the biological system, and not an artifact of preparation or assay technique, became clear in 1959 as Markert and Møller applied the zymogram method to lactate, malate and isocitrate dehydrogenases, esterases, alkaline phosphatases, and peroxidases from a number of tissues. Multiple molecular forms of the enzymes could occur in the same species, in the same organism, and even in the same tissue. Markert and Møller (1959) proposed that these forms be called "isozymes", and this term is now in widespread use. Lactate dehydrogenase was studied intensively over the next several years in a number of laboratories. Five distinct isozymes of LDH could be detected in mammals, there being a characteristic distribution of these in various tissues (Plagemann *et al.*, 1960; Vesell and Bearn, 1961). Any one of the isozymic forms could be dissociated into subunits with reagents that disrupt hydrogen bonding, such as urea or guanidine-HCl (Appella and Markert, 1961). There appeared to be two types of subunits, and the intact isozyme molecule was a tetramer. Two different subunits, taken four at a time in all possible combinations, could explain the five isozymes quite easily (Appella and Markert, 1961; Cahn *et al.*, 1962). There was much indirect evidence for this idea, and Markert proved it in 1963. He had found conditions under which the tetrameric enzyme could be dissociated into its constituent subunits, and then allowed to reassociate with the retention of activity. If the subunits are designated "A" and "B", Markert allowed a mixture of what were thought to be A_4 LDH and B_4 LDH to dissociate, and then reassociate at random. He predicted that if the two subunit hypothesis were correct, all five isozymes should appear, and in a predictable ratio, as indeed they did. For LDH, therefore, two different subunits, A and B, are used to construct five different tetrameric isoenzymes, which might be represented A_4, A_3B, A_2B_2, AB_3 and B_4. A review of the pioneering LDH work was given by Kaplan (1964).

Molecular multiplicity in a functional enzyme may come about in a number of different ways (Markert, 1968 and 1975). An enzyme consisting of a single polypeptide chain, coded for by a single gene, might exist in various forms because the genetic locus is multiple allelic. Two or more different genetic loci may control the synthesis of different polypeptide chains, which may function independently, or may polymerize to form a functional enzyme molecule. The number of combinations of functional polymers can vary. Activity may be associated with only one polymeric form, such as the tetramer, or more than one might be active, such as the dimer and the tetramer. Post-synthetic modifications of various kinds can also give rise to molecular heterogeneity. Proteolytic cleavage of a portion of a polypeptide chain could give rise to a population of cleaved and uncleaved molecules. Minor modification of a few residues of the polypeptide chain is possible. Association by the peptide with other molecules, such as carbohydrate or sialic acid units, would yield different populations of molecules. There might also be conformational differences in a set of molecules with the same primary and secondary structures. These differences might be the result of association of the peptide with small molecules or not, depending upon the molecular size of the functional unit and the number of subunits. Larger, more complex molecules could be expected to have more degrees of freedom in forming conformationally stable isozymes. It is quite likely that examples of all these devices could be found among the great variety of isoenzymes in nature.

A good sense of the progress in the isoenzyme field that was made in the 1960's can be gained by looking at the proceedings of the two major conferences sponsored by the

New York Academy of Sciences (Furness, 1961; Vesell, 1968). An excellent treatment of the biochemical genetics of human isoenzymes is given by Harris (1975).

The remainder of Unit VI is devoted to discussions of individual enzyme systems. Emphasis has been given to those which have medicolegal applications as genetic markers in populations. Isoenzymes which can be useful in identifying the tissue origin of a sample, or occasionally in other ways, such as in diagnosing pregnancy, were discussed in Unit II. The isozymes to be discussed in the present unit are controlled by polymorphic genetic loci, and are more or less useful in making distinctions in populations, depending upon the gene frequencies in the population of interest. Some of the isozymes can be reliably determined in bloodstains or in body fluid stains, while for others, techniques have not yet been devised. General principles of the medicolegal applications of genetic markers in identification and disputed parentage problems were discussed in section 18. The distributions of phenotypic frequencies in U.S. populations that could be located in the literature are given in tabular form, following the criteria outlined in the Preface. The abbreviations used to designate the isozymes follow Harris and Hopkinson (1976).

SECTION 27. PHOSPHOGLUCOMUTASE

27.1 Recognition of PGM

Phosphoglucomutase (PGM; EC 2.7.5.1; α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase) catalyzes the reversible conversion of glucose-1-phosphate and glucose-6-phosphate, glucose-1,6-diphosphate being required as a cofactor.

In 1936, Carl and Gerti Cori recognized a new glucose-phosphate ester in preparations in which frog muscle had been incubated with AMP and phosphate. This ester was converted to glucose-6-phosphate over time. The new ester was soon proven to be glucose-1-phosphate (Cori *et al.*, 1937) and it became clear that muscle tissue and yeast extracts contained an enzymatic activity responsible for the conversion of glucose-1-phosphate to glucose-6-phosphate. The enzyme was named phosphoglucomutase (Cori *et al.*, 1938a and 1938b).

27.2 PGM Polymorphism

27.2.1 *PGM*$_1$

In 1964, Spencer *et al.* found that red cell PGM from different persons gave three distinct patterns of isoenzymes following starch gel electrophoresis and specific histochemical staining (Spencer *et al.*, 1964b). Similar patterns could be observed in leucocyte, liver, kidney, heart or uterine muscle, brain, skin and placental extracts. Seven bands of activity were seen, and designated "a" through "g", the "a" being most cathodal. Only the "a" through "d" bands showed differences, and the simplest explanation was based on two alleles PGM^1 and PGM^2, conditioning the a and c, and the b and d bands, respectively. Studies on 133 families with 262 children showed no exceptions to this explanation. Many family studies support the two allele hypothesis of inheritance, including those of Monn (1969b), Wille *et al.* (1969), Renninger and Spielmann (1969) and Lamm (1970a). In 1965, Hopkinson and Harris found a family "Atkinson" in which variation in the e, f and g bands of PGM were observed. These bands were under the control of a second PGM locus. A subscript was used to designate the locus, the superscripts to designate the alleles. Thus, the PGM locus responsible for the a through d bands is PGM_1 with two common alleles, PGM_1^1 and PGM_1^2, giving rise to the three common phenotypes. Bands e through g are controlled by PGM_2. The majority of people are PGM_2^1 homozygotes, but occasionally, as in the Atkinson family, PGM_2^2 may be observed. People who were $PGM_2^1PGM_2^2$, i.e. Atkinson phenotype, were called Atkinson-1 if they were PGM_1 1, and Atkinson 2-1 if they were PGM_1 2-1. In addition, Hopkinson and Harris (1965) found five uncommon PGM_1 phenotypes in different families, best accounted for by the presence of three rare alleles of PGM_1^1 and PGM_1^2. These were called PGM_1^3, PGM_1^4 and PGM_1^5. All were observed as heterozygotes, PGM_1 3-1, 3-2, 4-1, 4-2 and 5-2. In 1966, Hopkinson and Harris found some further unusual phenotypes which were explained on the basis of two additional alleles, PGM_1^6 and PGM_1^7. PGM_1 6-1, 6-2, 7-1 and 7-2 patterns were seen in these studies. Harris *et al.* (1968) described PGM_1 8-1 and 8-2, adding PGM_1^8 to the list of rare PGM_1 alleles. Turowska and Gawrzewski (1979) found the PGM_1^9 allele segregating in a Polish family.

The rare phenotypes present many problems in comparing results from different laboratories. The only way to compare two samples properly is side by side, in the same gel, and this is not always possible. There are a number of rare phenotypes of PGM_1, particularly involving PGM_1^6 and PGM_1^8, whose relationships are not fully clear. An excellent discussion of the matter, with all the references, is given by Blake and Omoto (1975). Omoto and Harada (1970) and Shinoda and Matsunaga (1970a and 1970b) have reported several examples of PGM_1 8-1 in Japanese. Blake and Omoto (1975) compared several samples from Chinese, which had been diagnosed as PGM_1 8-1 with a reference PGM_1 6-1, which had been typed by Dr. Lie-Injo and Dr. Hopkinson. The samples were identical. This reference PGM_1 6-1 was then compared with a fresh specimen from the "PGM_1 8-1" subject of Omoto and Harada (1970). The samples were not identical, but very similar. The Japanese sample was not PGM 8-1 either, and it was proposed to call it PGM_1^{6JAPAN}. Further examples of it were found in the large population sample studied by Blake and Omoto (1975). In our own studies in New York, we found a peculiar PGM_1 phenotype in an Hispanic person, which was not quite PGM 6-1 nor PGM 8-1. We sent a part of our sample to Mr. B.G.D. Wraxall in London, who ran it against his reference PGM 6-1 and PGM 8-1 samples, and confirmed that it was neither of these. It did, however, match a sample in his collection which had been termed a PGM_1 "8-1 fast". We did not know of Blake and Omoto's work until after ours was at the printer (Mondovano and Gaensslen, 1975), but it seems quite possible that the London and New York samples might have been PGM_1^{6JAPAN} PGM_1^1. Blake and Omoto's (1975) paper should be consulted for further unusual variants at PGM_1.

So-called "silent" alleles have been described at the PGM_1 locus. They are very rare, but, if encountered, could lead to serious errors in disputed parentage cases. Fiedler and Pettenkofer (1969) found an individual without detectable PGM_1 isozyme activity. The father was PGM_1 1 and the mother, PGM_1 2. Their PGM isoenzymes had only about half the activity of normal samples of the same phenotype, and the authors supposed that these parents

were heterozygous for a silent allele, *PGM°*, which the propositus had inherited from each of them. Brinkmann *et al.* (1972a) found an apparent PGM_1 1 mother with an apparent PGM_1 2 daughter. If the samples were run on polyacrylamide gels, however, weak "a" and "c" band activity could be detected in the daughter's cells. Further studies showed reduced PGM_1 isoenzyme activity in both, and the allele responsible for the weak kind of PGM_1^1 was called PGM_1^{1r}. The mother was presumably $PGM_1^1PGM_1^{1r}$, and the daughter $PGM_1^2PGM_1^{1r}$. They said that Fiedler and Pettenkofer's subject might have been the same, since on starch gels, PGM^{1r} isozymes showed no activity. Ueno *et al.* (1976) reported a *PGM°* allele segregating in three generations of a Japanese family.

Until relatively recently, PGM_1 isoenzymes have been separated by electrophoresis on starch, agarose or cellulose acetate membranes, and occasionally on polyacrylamide gels. In 1976, Bark *et al.* examined red cell lysates for PGM_1 by isoelectric focusing on polyacrylamide gels over a pH 5–7 range, and observed 10 different electrofocusing patterns with samples from PGM_1 1, 2-1 and 2 sources. This was not a sulfhydryl effect. Four bands of activity, denoted "1−", "1+", "2−" and "2+" could be seen. PGM_1 1 types on starch could be 1+, 1− or 1+1− by isoelectric focusing, PGM_1 2 on starch could be 2+, 2− or 2+2−, and PGM_1 2-1 on starch could be 2+1+, 2+1−, 2−1+ or 2−1−. The ten phenotypes could be accounted for on the basis of four, rather than two, alleles at PGM_1. There was some family evidence presented in support of this view. The alleles were called PGM_1^{1+}, PGM_2^{1-}, PGM_1^{2+} and PGM_1^{2-}. Within starch phenotype PGM_1 1, the most frequent new phenotype was 1+1+ and the least frequent, 1−1−. Within starch phenotype PGM_1 2-1, 2+1+ was the most frequent, 2-1- the least frequent. Within PGM_1 2 on starch, 2+2− was observed in four people, 2+2+ in 2 others, the 2−2− not being seem in this sample of 123 persons.

Kühnl *et al.* (1977a) reported very similar results independently. Isoelectric focusing was carried out on commercially obtained ampholine-polyacrylamide gels with a pH range of 3.5 to 9.5, and agarose gel electophoresis could detect the differences as well. Leucocytes were the preferred material, though hemolysates and sperm extracts were also run. Isoelectric focusing was the preferred method. These results, like those of Bark *et al.* (1976) above, could be explained on the basis of four, rather than two, alleles at PGM_1, giving rise to ten phenotypes. Investigations on 15 families with 36 children confirmed that the four allele hypothesis correctly predicted the inheritance pattern. The alleles in this work were called PGM_1^{a1}, PGM_1^{a2}, PGM_1^{a3} and PGM_1^{a4}. Burdett and Whitehead (1977) carried out separations of PGM isoenzymes by isoelectric focusing, and these studies were extended by Sutton and Burgess (1978). The latter looked carefully at the PGM_1 isoenzymes in an isoelectric focusing system similar to that employed by Bark *et al.* (1976) in 101 unrelated people. The phenotypic patterns reported by Bark *et al.* (1976) were confirmed, and pedigree studies on 24 families with 52 children showed no exceptions to the pattern of inheritance assuming four alleles at PGM_1. Sutton and Burgess (1978) used the designations of Bark *et al.* (1976) for the genes.

Kühnl *et al.* (1977a) had used a somewhat different isoelectric focusing system in their studies, and they looked at leucolysates primarily where the other groups looked at hemolysates. It is difficult, therefore, to compare the phenotypic patterns. Kühnl *et al.* (1977a) reported gene frequencies of 0.6186 for PGM_1^{a1}, 0.1718 for PGM_1^{a2}, 0.1426 for PGM_1^{a3} and 0.0670 for PGM_1^{a4} in 291 persons from Hessen. Our calculations suggest frequencies of about 0.63, 0.11, 0.18 and 0.07 for PGM_1^{1+}, PGM_1^{1-}, PGM_1^{2+} *and* PGM_1^{2-}, respectively, for the 123 English people of Bark *et al.* (1976), and 0.62, 0.12, 0.14 and 0.12 for the 102 English people of Sutton and Burgess (1978). Bissbort *et al.* (1978) indicated that the 10 phenotypes of PGM_1 which could be seen by isoelectric focusing could be detected by acid starch gel electrophoresis. Tris-histidine buffers at pH 5.9 were employed with 18% starch gels. A new nomenclature system was introduced in this paper as well. The alleles were called PGM_1^{1F}, PGM_1^{1S}, PGM_1^{2F} and PGM_1^{2S}, and the phenotypes would be called 1F, 1FS, 1S, 1F2S, etc. This usage corresponds more closely to the nomenclature usually used for plasma protein polymorphic systems (Unit VII). "F" and "S" mean "fast" and "slow", and are meant to be descriptive of the electrophoretic mobilities. The correspondences between the original nomenclature of Bark *et al.* (1976), of Kühnl *et al.* (1977a) and the new system just described is: 1− = a3 = 1F; 1− = a1 = 1S; 2− = a4 = 2F; and 2+ = a2 = 2S. Guise (1979) examined the acid starch gel electrophoretic system described by Bisshort *et al.* (1978). He said that with certain modifications, the ten phenotypes could indeed be diagnosed, but that the method was quite cumbersome and that bloodstains could not be typed. The system was not regarded, therefore, as being a good one for PGM phenotyping in forensic serology.

27.2.2 *PGM_2*

As mentioned in the foregoing secton, 27.2.1, the Atkinson family revealed the fact that electrophoretic bands e through g were governed by a second locus, PGM_2 (Hopkinson and Harris, 1965 and 1966). Most people are homozygous for PGM_2^1, but this family had members who were $PGM_2^1PGM_2^2$, and who could be either PGM_1 1 ("Atkinson 1") or PGM_1 2-1 ("Atkinson 2-1"). A further phenotype. Palmer, was also reported, and appeared to disclose another allele at PGM_2, namely PGM_2^3. The propositus was PGM_1 1. Further alleles at PGM_2 have been described. PGM_2^4 was found by Monn (1968a) in a healthy Italian male who appeared to be PGM_1 1, PGM_2 4-1. PGM_2^4 and PGM_2^5 were described by Harris and Hopkinson in a personal communication to Giblett (1969) and published by Hopkinson and Harris (1968 and 1969a). Monn (1969a) found what he thought was a PGM_2^5 pattern in a Norwegian Lapp, but the specimen could not be compared with known standards. Further examples of the Atkinson phenotype were found in San Francisco by Lie-Injo (1966), in Michigan

by Brewer *et al.* (1967) and in Seattle by Giblett (1967). All these Atkinson phenotypes, including those of the original family, occurred in Black people. Atkinson phenotype can, however, occur in Caucasians. Gordon *et al.* (1968) reported such a person in Capetown. They also reported what they thought was a PGM_2 2 homozygote in a Black person in Capetown, and called the phenotype "PGM Capetown". Giblett (1969) described an apparent PGM_2^2 homozygote from Mozambique, who was PGM_1 2-1. The Giblett (1969) and Gordon *et al.* (1968) "PGM_2 2" patterns, Mozambique and Capetown, do not look quite alike in the photographs.

In 1969, Santachiara-Benerecetti and Modiano found a variant at the PGM_2 locus in pygmies, which was provisionally called PGM_2^{Pyg}, but which, they said, could become PGM_2^6 (Santachiara-Benerecetti and Modiano, 1969a). Meanwhile, Monn and Gjønnaess (1971) found a PGM_2 locus variant, and suggested that it be called PGM_2^6. Santachiara-Benerecetti *et al.* (1972b) found another example of their PGM_2^6 in an Indian population. It differed slightly in activity from the pygmy phenotype, and they said that these would be distinguished as PGM_2^{6Pyg} and PGM_2^{6Ind} for the time being. They said that the Norwegian variant of Monn and Gjønnaess (1971) should be promoted to PGM_2^7. In this same Indian population was found a further variant type, and the gene responsible for it was named PGM_2^8.

In 1973, Kirk *et al.* reported a number of "PGM_1 5-1" types in an Asian-Pacific population. Studies on other populations in the area, however, led them to conclude that this was, in reality, a second locus variant which should be designated PGM_2^9 (Woodfield *et al.*, 1974; Blake and Omoto, 1975). Blake and Omoto (1975) found a number of other people with yet another PGM_2 phenotype, accounted for by a new allele in the series, PGM_2^{10}.

27.2.3 *PGM*$_3$

In 1968, Hopkinson and Harris found a third group of isoenzymes controlled by yet another PGM locus. In the original studies, as mentioned above, the PGM_1 and PGM_2 isoenzymes could be found in a variety of tissues other than red cells. In placenta, the phenotype was found to be that of the baby rather than that of the mother, and placental tissue clearly reveals the third set of isozymes. They are detectable in other tissues as well, but not in red cells. Studies of dizygotic twins showed that two common alleles at the PGM_3 locus, PGM_3^1 and PGM_3^2, can give rise to three common genotypes (Hopkinson and Harris, 1968; Harris *et al.*, 1968). Lamm (1969) found that PGM_3 isozymes could be detected in leucocytes. The concentration of PGM_3 isoenzymes is low, however, representing but a small part of total PGM activity, and PGM_3 locus typing usually requires that more material be applied to the gel. Family studies by Lamm (1969) gave results consistent with the two allele hypothesis for PGM_3. The PGM_3 locus is not closely linked to either of the other two PGM loci. Where the gene frequencies for PGM_1^1 are of the order of 0.75 in British people, and only slightly greater in most Black populations studied, those for PGM_3^1 differ significantly between the two groups. Hopkinson and Harris (1968) found 0.74 for British populations and 0.34 for Nigerians. Lamm (1970a) found PGM_3^1 to be about 0.75 in 1,031 unrelated Danes.

Electrophoretic patterns of many of the phenotypes are indicated diagramatically in Fig. 27.1. According to the data of Harris *et al.* (1974), the incidences of PGM_1 alleles other than PGM_1^1 and PGM_1^2 are very low in European Caucasians, of the order of 1 or 2 in 10,000 at most.

27.2.4 Linkage relationships of the PGM loci

The data of Hopkinson and Harris (1966) and of Parrington *et al.* (1968) indicate that none of the PGM loci are closely linked to one another. The study of linkage relations between blood group, isoenzyme, serum protein and other marker characteristic loci is moving forward at a very rapid pace. A review of the literature of all established and suspected linkages would be too involved, and the interested reader is referred to the specialized literature. McKusick (1975) and McKusick and Ruddle (1977) contain considerable information, and Figure 1.36 may be consulted for a tabular summary.

Generally speaking, there are two major approaches to human chromosome mapping. Studies of clones of human-hamster or human-mouse cell hybrids may be used to localize a genetic locus on a particular chromosome or chromosome group. Provided that a suitable reference marker is available on that chromosome, linkage analysis can then be carried out using pedigree data from informative matings. For a review of the methods, which can become quite involved, see Renwick (1971b). There is considerable information, too, in Chapter 27 of Race and Sanger (1975).

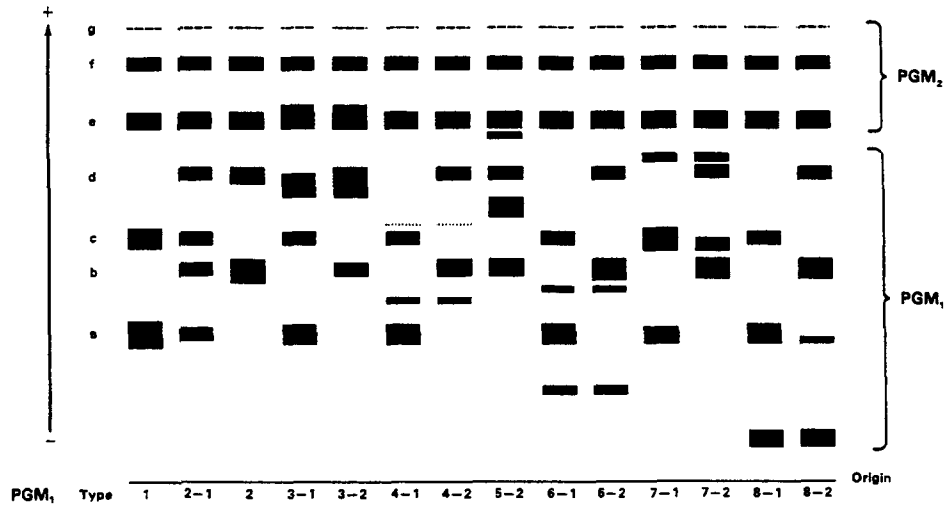
The *PGM* loci do not appear to be linked to one another nor to most other blood group, isoenzyme or serum protein marker loci (Parrington *et al.*, 1968; Lamm, 1970a; Lamm *et al.*, 1970). PGM_1 is linked to Rh and to PGD (Renwick, 1971a). Hybrid cell studies have shown that PGM_1 is on chromosome 6 (Jongsma *et al.*, 1973) and PGM_2 has been assigned to chromosome 4 (see McKusick, 1975, catalog no. 17200).

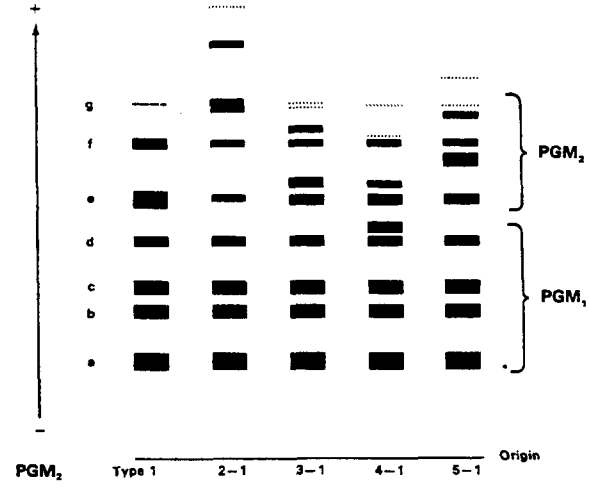
As has been discussed to some extent in section 1.2.4.3, linkage of loci means that they are located on the same chromosome, and have a crossover frequency of less than 50%. Renwick (1971b) used the term "synteny" to mean that loci are on the same chromosome, but are *not* "linked" because they are too far apart. The term is now widely used, and helps to make the term "linkage" more precise. Linkage is detected by studying crossover frequencies in the offspring of informative matings. The situation is complicated somewhat by the fact that the crossover frequencies between the same two loci may differ in the two sexes.

27.3 Biochemical Studies on PGM Isoenzymes

27.3.1 Properties of the PGM enzyme

PGM is ubiquitous, occurring in many cells of animals, plants and microorganisms. The rabbit muscle enzyme has





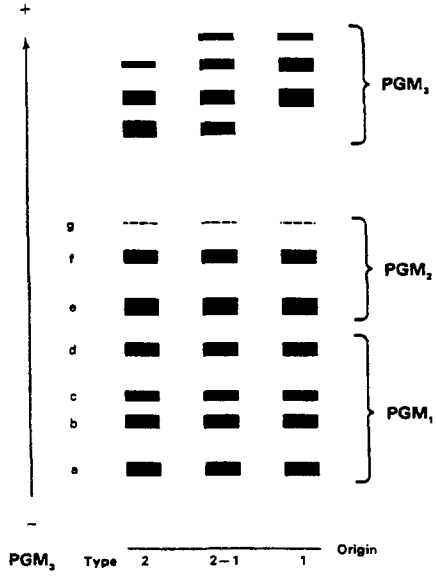


Figure 27.1

Electrophoretic Patterns of PGM Phenotypes

been particularly well studied. The natural substrate for PGM is glucose-1-phosphate (Glc-1-P), although the enzyme also catalyzes the reversible interconversion of C-1 and C-terminal phosphate groups of a number of sugars, including pentoses, though less efficiently. PGM exists in so-called "phospho" and "dephospho" forms, and these are separable. The enzyme requires Glc-1,6-diP and Mg^{++} for activity. It has been known for quite some time that serine is a component of the active site, and that phosphate is attached to the -OH group of serine in the phosphoenzyme (see in Handler *et al.*, 1965). Earlier studies on PGM, with documentation, were well reviewed by Najjar (1962).

PGM is activated by Mg^{++} and Glc-1,6-diP. Much of the data on activators and inhibitors of PGM is contradictory, and difficult to understand, particularly the work on "activation" by chelating compounds. Metal chelating agents, such as imidazole, appear to activate PGM (see Harshman *et al.*, 1965; Robinson *et al.*, 1965), though it now appears that the enzyme is very sensitive to inhibition by heavy metals, such as Zn^{++}, and that the imidazole works by removing these ions. In the absence of excess Mg^{++}, chelating agents can inhibit (Zwarstein and van der Schyff, 1967). The enzyme from rabbit muscle, at least, has six -SH groups, and these can be titrated with -SH reagents at a faster rate in the dephosphoenzyme than in the phosphoenzyme (Bocchim *et al.*, 1967). Oxidation or blocking of the -SH groups significantly reduces enzymatic activity. PGM is inhibited by a number of organic and inorganic anions. It has been known for quite some time that the phosphoenzyme reacts with glucose monophosphates, but not with Glc-1,6-diP, while the opposite is true of the dephosphoenzyme. Kinetic and ^{32}P transfer experiments have indicated that free Glc-1,6-diP is not formed in the reaction as an obligatory intermediate, and that dephosphoenzyme is not formed in every catalytic cycle (Ray and Roscelli, 1964; Goumaris *et al.*, 1967). These and other findings are consistent with a mechanism in which phosphoenzyme-glucose monophosphates are in dynamic equilibrium with dephosphoenzyme-Glc-1,6-diP. The dephosphoenzyme-Glc-1,6-diP may be regarded as the intermediate, capable of dead-end dissociation (Ray and Peck, 1972). A simplified scheme for the reaction, as given by Ray and Peck (1972) is shown in Fig. 27.2. The more recent biochemical work on PGM was well reviewed by these authors.

Purified examples of PGM exhibit molecular heterogeneity. The molecular species have been separated both by column chromatography and by electrophoresis (Joshi *et al.*, 1967; Dawson and Mitchell, 1969). Electrophoresis appears to give better separation. These forms are called "isoenzymes" by some workers, and this usage is certainly not incorrect. However, for those of us accustomed to thinking of isoenzymes as heterogeneous molecular species determined by different genes, a distinction is perhaps helpful. Dawson and Green (1975) have shown that the molecular heterogeneity appears to result from single unit charge changes in the same molecule, probably involving the sulfhydryl residues. The behavior is characteristic of PGM from many sources (Dawson and Jaeger, 1970). Dawson and Green (1975) found that successive alteration of the six free -SH groups in PGM results in systematic changes in the "isoenzyme" pattern. Blackburn *et al.* (1972) found that rabbit muscle phosphoglucose isomerase exists in different forms of this kind as well, and used the term "pseudoisoenzymes" to describe them.

27.3.2 Studies on the enzymes produced by the different PGM loci

The evidence suggests that the isoenzymic products of any particular locus are very similar in their properties. Modiano *et al.* (1970) found that the average PGM activity values of cells representing a number of people in each of the three common PGM_1 phenotypic classes were very close. Densitometric studies on the isoenzymes conditioned by PGM_1^1 and PGM_1^2 in all three phenotypes also indicated that there were no detectable differences (Terrenato *et al.*, 1970). This conclusion appears to be supported by the thermal denaturation studies of McAlpine *et al.* (1970a) who found no differences in the isoenzymic products of the alleles at any one of the loci. There were, however, differences in thermostability among products of the different loci, the isozymes of PGM_2 being most stable, those of PGM_3 least so, with PGM_1 products in between. The relative contribution of the isoenzymes governed by the three loci to the overall PGM activity in various tissues was examined by McAlpine *et al.* (1970b). In the majority, the PGM_1 isoenzymes accounted for more than 80% of the activity. In liver and cardiac muscle, the figure was 95%. PGM_1 isoenzymes in the red cell account for about 50% of the activity. PGM_2 enzymes represented the second largest fraction of total activity in most tissues. Fibroblasts in culture were an exception. Red cells were most active, PGM_2 isoenzymes accounting for about 50% of the activity. PGM_3 enzymes make a very small contribution to the total activity of all tissues studied, except cultured fibroblasts, where they contributed about 6.5% slightly more than did PGM_2 locus products. PGM_3 products are not detectable in red cells by the usual starch gel electrophoretic procedures.

Molecular weights of the PGM isoenzymes from human tissues were estimated by McAlpine *et al.* (1970c) by gel filtration chromatography to be about 51,000 for PGM_1, 61,000 for PGM_2 and 53,000 for PGM_3. The human muscle enzyme, purified many fold, has a molecular weight of about 60,000 as estimated by its sedimentation in the ultracentrifuge (Joshi and Handler, 1969). Santachiara Benerecetti and Modiano (1969b) estimated a MW of 71,000 for PGM_1 isoenzymes by sedimentation ultracentrifugation, and that data indicated that the PGM_2 isozymes might differ very slightly. The rabbit muscle enzyme MW has been estimated at 62,000–67,000 by different methods (Ray and Peck, 1972).

Fisher and Harris (1972) found that additional isoenzymes appeared in material from red cells, lymphocytoid cells or placenta, and seemed to be present in direct proportion to the *in vivo* age of the proteins. They could be found

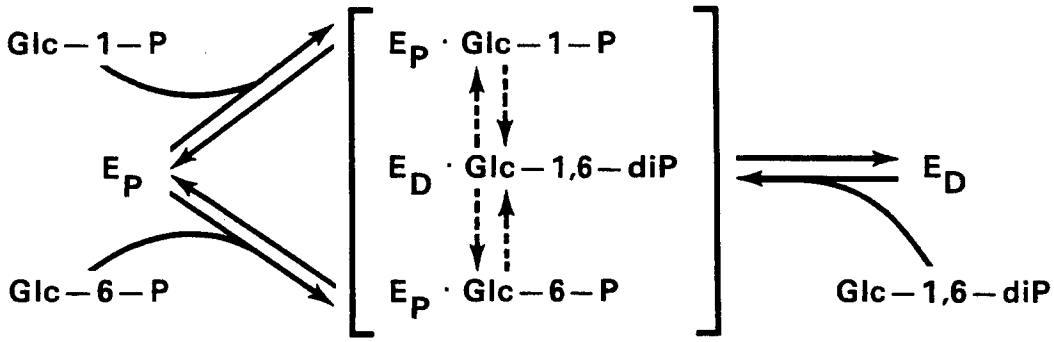


Figure 27.2 Scheme for the Phosphoglucomutase Reaction.

Abbreviations: Glc—1—P = glucose—1—phosphate; Glc—6—P = glucose—6—phosphate; Glc—1,6—diP = glucose—1,6—diphosphate; E_P = phosphoenzyme; E_D = dephosphoenzyme

associated with the products of all three *PGM* loci and represented molecules more negatively charged than the usual isoenzymes. These were called "secondary" isoenzymes. The phenomenon was not thought to be attributable to changes in sulfhydryl groups. Storage changes were noted in the PGM_3 isozymes from placenta. These changes affected both the primary and secondary isoenzymes. Such changes were not seen with PGM_1 and PGM_2 products, but could be produced by sulfhydryl reagents.

Turner *et al.* (1975) looked at PGM patterns in red cells of varying average age, and noted that there was a consistent increase in the c and d bands of the PGM_1 locus at the expense of the a and b isozymes as the cells aged. Similarly, the f and g bands of PGM_2 increased at the expense of band e. These observations are consistent with, but do not prove, the idea that the c and d isozymes of PGM_1 and the f and g isozymes of PGM_2 represent post-synthetic alterations of the primary gene products. In this view, PGM_1^1 codes for the a band protein, PGM_1^2 for the b, and PGM_2^1 for the e.

Quick *et al.* (1972) noted that the enzymes of the PGM_2 locus had considerable phosphopentomutase (PPM) activity, as defined by catalysis of the reversible conversion of deoxyribose-1-phosphate and deoxyribose-5-phosphate. PGM_1 and PGM_3 enzymes had PPM activity as well, but it was much lower. Quick *et al.* (1974) enlarged these studies. The K_m for Glc-1-P was dependent upon the Glc-1,6-diP concentration. PGM_1 isozymes were most efficient at [Glc-1,6-diP] of less than 10 μM. The K_m of PGM_2 enzymes for Glc-1-P decreased as a function of increasing [Glc-1,6-diP], especially over the 100–400 μM range. The K_m of PGM_1 or PGM_2 enzymes for ribose-1-P was unaffected by Glc-1,6-diP concentration. Ribose-1-P competitively inhibited the phosphoglucomutase activity of all the isozymes.

27.4 Medicolegal Applications

27.4.1 Disputed parentage

The PGM_1 isoenzymes have been used for some years in some laboratories in cases of disputed affilitation (Monn, 1969c; Herbich and Pesendorfer, 1969; Kneiphoff and Nagel, 1970; Halasa (1977); Boorman *et al.*, 1977). The chances of excluding a true nonfather by PGM_1 is of the order of 14% in western European populations. Polesky *et al.* (1976), said that the chances of excluding a falsely accused Caucasian father with the PGM_1 system is 14.21%, while for a falsely accused Black father, it is 11.86%. These figures are applicable to U.S. populations, and are similar to those quoted in Dykes and Polesky (1978) and in Chakraborty *et al.* (1974).

The possibility of encountering a silent allele of PGM_1 should not be overlooked. While rare, such alleles do exist (Fiedler and Pettenkofer, 1969; Brinkmann *et al.*, 1972; Ueno *et al.*, 1976), and would, if overlooked, lead to incorrect interpretations in parentage investigations. Silent alleles of PGM_1 were discussed in section 27.2.1.

Welsh *et al.* (1979) have shown that the expected increase in exclusion probability in parentage cases, using isoelectric focusing for PGM_1 phenotyping, is realized in practice. The chances of excluding a true nonfather are about 25% with the 10 phenotypes in western Europeans.

Chen *et al.* (1977) have recently shown that PGM_1 patterns are fully developed in fetal blood.

27.4.2 PGM grouping in bloodstains

27.4.2.1 Development of methods. In 1967, Culliford reported that dried bloodstains could be successfully typed for the PGM_1 isozymes. The method was the original one of Spencer *et al.* (1964b). Horizontal starch gel electrophoresis was performed for about 17 hours at 5° using 0.1M Tris, 0.1M maleic acid, 0.01M EDTA and 0.01M $MgCl_2$ adjusted to pH 7.4 with NaOH as bridge buffer, and a 1:10 dilution of this solution as gel buffer. Gels were sliced horizontally following electrophoresis, and overlaid with a 1% agar gel containing Glc-1-P, Glc-1,6-diP, $MgCl_2$, NADP, Glc-6-P dehydrogenase, PMS and MTT. The original workers had applied the "activity stain" to the gel on filter paper. The activity stain for PGM is based on the reaction sequence indicated in Fig. 27.3. NADPH generated in the reaction sequence will reduce the MTT-tetrazolium dye to an insoluble formazan in the presence of PGM. It may be noted that Glc-1,6-diP is required in catalytic quantities, and that most examples of less than highly purified Glc-1-P contain sufficient amounts of it as an impurity. Wraxall and Culliford (1968) soon found that the starch gels could be made "thin" to begin with (about 1 mm), and that enzyme typing on these was far more convenient because they did not have to be sliced prior to application of the detection mixture. The technique is fully described by Culliford (1971). The second locus phenotypes could be determined as well, but the isozymes determined by PGM_2 were found to be not as stable as those of PGM_1. The former were detectable in bloodstains a week to two old. PGM_1 isozymes were generally determinable in stains a month old, and sometimes to as much as three months. Occasionally, much fresher stains are found to be untypable too.

There have been many technical modifications of the original procedure, and a number of supporting media other than starch have been employed for the determination of PGM isoenzymes. Brinkmann and Fritz (1968) described a buffer system for PGM consisting essentially of the original buffer components, but adjusted to pH 7.2. The gel was made up in a 1:9 dilution of bridge buffer, adjusted to pH 7.5. This system was used to group PGM in bloodstains (Brinkmann, 1969). Oepen (1970) found the Culliford (1967) system entirely satisfactory for bloodstains, except that the buffer system of Radam and Strauch (1969) was used. These latter described a discontinuous buffer system for PGM consisting of Tris-acetic acid, pH 7.4, for bridge buffer, and a 2.9 mM phosphate buffer, pH 7.2, for the gel. This, they said, gave lower current flow and less heating during the electrophoretic run.

Horizontal polyacrylamide gels have been employed for typing PGM and other isoenzymes as well. Wrede *et al.* (1971) reported successful PGM_1 typing in such a system using the original buffer system of Spencer *et al.* (1964b) for the anodic tank and 0.33M phosphate buffer, pH 6.2 for the cathodic. Gel buffer was 10 mM phosphate, pH 6.9. Further details were given by Hoppe *et al.* (1972). Some polymerization initiators and catalysts (see in section 2.3.7.1) can inhibit some of the enzymes. Notably, TEMED inhibits red cell acid phosphatase (section 29.5.3), and it was found best to polymerize the gels with 3-dimethylaminopropionitrile (DMAPN), potassium ferricyanide and ammonium persulfate. The gels were soaked a long time in water, and then in gel buffer prior to use, this procedure serving to have gels ready when they were required as well as to wash out excess monomers and initiators which might inhibit enzymatic activity. This system was applied to red cell lysates, rather than to bloodstains.

Cellulose acetate foils have been used as well. Sonneborn (1972) reviewed his own work, as well as that of some others, in the development of cellulose acetate membrane (CAM) methods for a number of enzymes, including PGM. References to his earlier papers are cited. The methods were developed for lysates. Raszeja and Miscicka (1976), however, said that they had obtained very good results using a slightly modified Sonneborn method for bloodstains. Miscicka *et al.* (1977) reported good results with Cellogel CAM foils for PGM in bloodstains. Grunbaum (1974) developed a microprocedure for PGM on CAM using the Beckman microzone system. Eight samples could be run simultaneously. Zajac and Sprague (1975) said that this system gave good results with bloodstains.

Agar and agarose have been used as electrophoretic support media for the separation of PGM isozymes. Monn

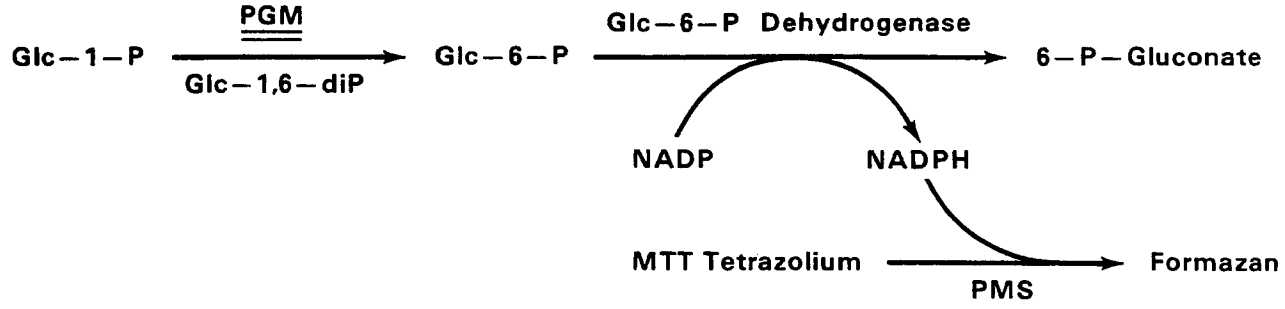


Figure 27.3 Detection Reaction Sequence for PGM

(1968b) looked into this type of system in a systematic way. He preferred Reinagar to the agarose and Bactoagar that were tried, and the gels were made to 1% concentration on microscope slides. The most desirable gel buffer was 2.7mM monobasic potassium phosphate and 1.3 mM dibasic sodium phosphate, ionic strength 0.01, pH 7.4. Twice this concentration was used for the bridge buffer, and the system was recommended for routine lysate typing. Kneiphoff and Nagel (1970) employed this system in their work. Agarose-acrylamide gels have been used on a preparative scale to separate the products of the three PGM loci (McAlpine *et al.*, 1970b).

The isoelectric focusing systems discussed in section 27.1.1, whose application to PGM_1 isozyme typing suggested the four allelic gene hypothesis, are applicable to bloodstains (Bark *et al.*, 1976; Sutton and Burgess, 1978). With 10 phenotypes, instead of the three seen on other support media, the discrimination index for PGM_1 is significantly increased. Application of procedures capable of detecting the ten (rather than three) phenotypes is often referred to as "PGM_1 subtyping".

There are a number of reports describing what may be called multiple systems, in which more than one polymorphic enzyme or protein is determined in one and the same electrophoretic run. Such systems may be devised in a number of ways. Buffer systems can be found in which the products of several independent isozyme system loci are separated on a gel, and can then be detected separately. If thick starch gels are used, and sliced before staining, there are two gel surfaces to work with. Some of the enzymes can be detected with substrates which yield a fluorescent product. Gels can sometimes be incubated first with these substrates, and examined for the phenotypes under long wave UV light, and then stained for other enzymes with filter paper or gel overlays. Hummel (1970) combined PGM with EAP on one gel, and ADA with AK on a second. Wrede *et al.* (1971) could detect PGM_1, ADA and AK isozymes on one 30 cm long acrylamide gel. Martin and Niebuhr (1971) did the same with PGM, ACP, AK and ADA. Gussmann and Rames (1972) combined PGM and GPT typing on one gel, and Neilson *et al.* (1976) reported a system for the simultaneous detection of PGM, AK and Peptidase A. Wraxall and Stolorow (1978) have devised a system for determining PGM, ESD and GLO on one plate, and the procedure is applicable to bloodstains as well as to lysates.

Weidinger and Schwarzfischer (1980) reported on a procedure for PGM_1 subtyping using agarose gel isoelectrofocusing (AGIF) for hemolysates. All ten types were readily determinable using this system.

Shaler and Dhawan (1980) recently reported that the PGM_1 subtypes could be determined by conventional electrophoresis on agarose gels (1%) using 5.7mM phosphate-2.5mM citrate gel buffers and 0.29M phosphate-0.1M citrate bridge buffers, both at pH 5.6. This system was suggested as an alternative for laboratories not set up for isoelectric focusing. Bloodstains could be typed using the system. They have also found (Dhawan and Shaler, 1980) that this electrophoretic system is suitable for the simultaneous determination of PGM_1 subtypes and Gc phenotypes (section 41).

Marbach (1980) carried out a series of experiments in which the concentration of PMS in the overlay detection mixture used for visualizing PGM isozymes was varied. It is well known that the gel "background" slowly darkens upon incubation with PMS- and MTT-containing overlays because of the light sensitivity of the system. Most authors have recommended PMS concentrations ranging from 0.16 to 0.65mM. Marbach (1980) said that PMS concentrations of 0.016mM gave clearer results in PGM typing, and allowed for longer incubation periods than were possible with the higher PMS concentrations.

Goetz and Baxter (1980) reported a most interesting case in which two blood specimens (including stains), which were identical in ABO, Rh, MN, Duffy, Kidd, Lutheran, Xg(a), Hp, AK, ADA, ACP, ESD, GLO and conventional PGM types, were discriminated by PGM_1 subtyping.

27.4.2.2 Survival of PGM isoenzymes in blood. Most systematic studies on the survival of PGM or other markers in dried blood are carried out on experimental bloodstains. Such experiments yield very useful data, but the fact remains that examiners seldom have any control over, or knowledge of, the conditions characterizing the history of a bloodstain submitted in a case. Many factors affect the retention of enzymatic activity, and little is known about the detailed way in which these influence the proteins. As a result, occasional examples of quite fresh stains may be found which are without activity, and some rather old stains may yield satisfactory results. A related problem is that of extractability. If the protein cannot be induced to enter the gel or cellulose acetate membrane, the sample is not groupable even though the enzyme might still be active. The presence of inhibitors as contaminants is equally possible. There is also some apparent variability in the survival characteristics of an enzyme depending upon the electrophoretic and activity-detection systems used.

Culliford (1971) said that PGM_1 isozymes were usually determinable in four week old stains, and in occasional stains up to 12 weeks old. Brinkmann (1969) said that bloodstains on absorbent materials were typable for up to 7 weeks, and dried blood on harder substrata, which could be scraped off, could be typed for PGM for up to 12 weeks. Oepen (1971) could group stains 8–10 weeks old in most cases, and occasionally a stain 20 weeks old. Herzog and Sobotka (1972) typed bloodstains 9 weeks old. Welch (1972b) found that PGM was determinable in 4 week old bloodstains, at which point the experiment ended. Rothwell (1970) found that PGM was typable in some stains up to 5 months old, while others gave unreadable results at 2 months age. About half the stains were typable at 3 months age, 16% at 5 months, and by six months, activity was virtually gone in all samples. Turowska (1971) said that 2 month old bloodstains could be typed reliably. Denault *et al.* (1978) looked at the detectability of PGM isozymes in bloodstains

on glass, cotton, nylon, wool, perma-press material and denim under high and low humidity storage conditions. In most cases, the stains could be phenotyped for up to 26 weeks. Under some conditions, the phenotypes could be determined at 13 weeks, but not at 26 weeks. High humidity tended to increase the rate at which the enzymes became undetectable, and PGM was detected less often in bloodstains on perma-press and denim at 26 weeks than on other substrata. Several reports indicate that PGM in fairly old stains is possible on CAM. Raszeja and Miscicka (1976) and Miscicka *et al.* (1977) said that six month old stains were routinely determinable, and a type was obtained from one stain that was 2-1/2 years old. Zajac and Sprague (1975) could type stains 20 months old in their studies.

Herzog and Sobotka (1972) found that 90% of samples standing at room temperature for 106 days could be grouped for PGM. With post-mortem samples, they said that results could sometimes be obtained with bloods taken more than 4 weeks after death. Rothwell (1970) found that frozen lysates were typable for PGM for about 18 months with rare exceptions, but that only about half the samples were still active after 2 years. Rees *et al.* (1975) examined the PGM types in blood specimens collected for blood alcohol determination after various amounts of time at room temperature. The containers have NaF and potassium oxalate in them so as to yield final concentrations, when filled, of about 1% and 0.25%, respectively. The containers were only filled to about 2/3 capacity in the studies. Some samples could be correctly typed for PGM when 72 days old, but some failed to give results at 44 days. With older samples, there were some apparent changes in phenotype, which will be discussed below. All the samples could be correctly typed for up to 32 days.

It may be noted that PGM appears to be stable almost indefinitely in lysates kept in liquid nitrogen (Culliford, 1971; and our own observations).

27.4.2.3 Problems in PGM grouping of blood. Most of the time, PGM_1 enzymes can be typed in relatively fresh stains without difficulty. In older stains, there is sometimes development of a diffuse band in the d band region, which can cause difficulty in typing (Culliford, 1971). Older specimens of whole blood, or post-mortem samples can show considerable enhancement of activity, leading to large diffuse bands. This effect is usually accompanied by bacterial infection of the sample (Culliford, 1971). We have observed such patterns in post-mortem samples on a number of occasions.

The only reports on actual misgrouping because of sample age have been concerned with stored whole blood, rather than with dried stains. Brinkmann (1974) noted that samples containing NaF, and standing for up to a year at 4°, could undergo apparent phenotypic changes. In eight samples, out of 58 studied, PGM "2-like" patterns were seen in samples that were PGM 1 or PGM 2-1 when fresh. Brinkmann thought that the fluoride might be affecting the enzyme. Culliford (1971) noted that the presence of fluoride in a sample can cause considerable distortion of the PGM band pattern, but this was primarily an ionic strength effect. Rees *et al.* (1975) found two alterations in their study of the blood alcohol samples, which had been kept at 4°, and contained NaF. In one case, a PGM 2-1 gave correct results for 32 days, no result at 44 days, and then a PGM 2 pattern at 51 days. In another, a PGM 1 sample, correctly grouped at 32 days, was negative at 44 days, and gave a PGM 2-1 result at 51 days. It would appear that considerable caution should prevail in interpreting PGM patterns from these kinds of samples.

27.4.3 PGM grouping of semen, vaginal secretions and other tissues.

Culliford first reported the detectability of PGM_1 isozymes in seminal plasma in 1969 at the 5th International Meeting of Forensic Sciences in Toronto. Culliford (1971) discussed the subject in his book as well. Around this same time, Renninger and Sina (1970) noted that PGM_1 enzymes may be determined in spermatozoa, and this observation has since been confirmed in a number of laboratories (Brinkmann and Koops, 1971; Erickson, 1974; Blake, 1976; Blake and Sensabaugh, 1976). The PGM_1 isozymes, as determined by thin starch gel electrophoresis, were the same in seminal plasma as in red cells lysates from the same individual, although the concentration of the enzyme was lower in seminal plasma, and more material was required (Culliford, 1971). It was found, too, that detectable PGM_1 enzymes may be seen in vaginal secretions as well. In sexual assault cases, therefore, the victim's PGM_1 type must be known before any typing result from a vaginal swab that contains semen can be interpreted. Red cell and seminal plasma PGM phenotypes in the same person were always the same, and this fact has been confirmed by Rees *et al.* (1974). Radam and Strauch (1971) confirmed that PGM_1 phenotypes can be detected in seminal stains, and that cervical mucus obtained post-mortem had considerable PGM_1 activity.

In 1975, Rees and Rothwell carried out a study on the determination of PGM_1 isoenzymes in seminal stains and in post-coital vaginal swabs. Seminal stains, made from semen samples of known age, were examined for PGM a number of times up to a stain age of 35 days. 30 stain specimens from 28 individuals were included, and seminal plasma type was the same as red cell lysate type in every case where the comparison could be done. Two seminal plasma specimens had no detectable PGM activity. Eight stain specimens were inactive or untypable at 2 days, although prepared from active semen. In one peculiar case, a PGM 2-1, the specimen was untypable at 2 days, inactive at 8 days, but correctly typed at 15 days. From the data, it appeared that loss of activity in seminal stains upon aging occurs at a faster rate than in bloodstains. There was one discrepant result. A PGM 1 stain grouped as a PGM 2-1 at 7 days, but was correctly grouped at 17 days. It should be noted that the seminal samples had in some cases been stored frozen after collection and prior to making stains. Sixty-two post-coital swabs from 37 persons were examined for PGM. The

amount of semen present was assessed by sperm density, and qualitative acid phosphatase tests. As many as three swabs were taken and tested but not with every subject. In 21 of 37 "first swabs", no PGM activity could be detected. In 12 of the remaining "first swabs", the PGM type was identical to that of the woman's red cells, although in the case of seven of the couples, the PGM types were the same in both people. Discrimination of PGM isoenzymes of seminal origin is possible only in cases where the woman is homozygous, and components representing the product of the other allele are found. Taking several swabs appeared to be useful, since in a few cases results were obtained with a second and/or third one, while the first swab was negative. In some cases, the swabs contained blood, which could account for the results obtained. There were four instances of discrepant results. In two, the swab type differed from that of either partner. In another, a swab from a PGM 1 woman with a PGM 1 partner grouped as PGM 2 on the first swab, but as PGM 1 on two further swabs. In one case, a pair of swabs was grouped as PGM 2-1 from a woman whose red cells may have been PGM 4-1, and who had a PGM 1 partner. Rees and Rothwell (1975) said that seminal stain PGM grouping appeared to be reasonably worthwhile, but that much difficulty was associated with PGM typing of vaginal swabs.

In 1976, Price *et al.* presented the results of another extensive study of PGM typing in seminal plasma, seminal stains and post-coital vaginal swabs. Semen could be grouped reliably, and loss of activity was temperature dependent, being greatest at 37°, least at −15°. Seminal stains were active for 12 days when stored at room temperature, but at 37°, activity was lost within 24 hours. Vaginal swabs were examined from two groups of subjects, one in which no semen was present in any of the swabs, and the other in which swabs were taken post-coitally. 471 semen-free swabs from seven subjects over the course of 33 menstrual cycles were examined for PGM activity. 27% of these had readable PGM isoenzymes. Between 25% and 30% of these showed activity only in the first few days of the menstrual cycle, and the activity was thought to be due to the presence of blood. Other donors showed sporadic activity around midcycle, or else more or less continuous activity with a gap around midcycle. In all, 17% of the swabs examined showed PGM activity attributable to vaginal secretions rather than blood. In the post-coital swab part of the study, 769 samples were examined, and 471 of these were semen-free. Of the remaining 298 swabs from subjects who were sexually active during the course of the study, about half (158) were found to have semen present. Of these 158, 59 had readable PGM activity. It can happen that swabs with high semen density, as judged by sperm cells or acid phosphatase activity, do not yield readable PGM. Experiments on the artificial mixing of semen of known PGM type with semen-free swabs, and incubation of these swabs at 37° in a moist environment, indicated that PGM activity was lost within about 6 hours. The length of time between the deposition of semen and collection of the swab was regarded as the most critical factor in determining whether or not a typable PGM would be observed. It was found that inexperienced readers of PGM plates can sometimes misinterpret the band patterns, but experienced readers did not make errors. It was concluded that, under favorable circumstances, there is no reason not to attempt PGM typing in swabs, provided an electrophoretic system is used which gives a clear b and c band separation, and that care is taken with the interpretation of the patterns obtained. Only experienced readers, it was said, should be allowed to read and interpret PGM plates for judicial presentation. The potential value of PGM typing in vaginal swabs in cases of sexual assault was briefly discussed by Willott (1975).

Eastwood (1977) reported some experiences with the PGM typing of vaginal swabs. In many samples, a "fast" band of PGM activity was seen with swabs, and in semen-free swabs this band was much clearer than the bands due to the first and second PGM loci. The band was seen in semen-positive swabs as well. PGM activity in vaginal swabs was greater in samples collected from sexually stimulated women than otherwise. Tanton (1979) was unable to confirm any correlation between vaginal PGM patterns and degree of sexual stimulation. White *et al.* (1978) recently noted that, in grouping 52 semen-positive vaginal swabs from sexual assault cases, the results were very frequently identical to the victim's PGM type. It is unreasonable to imagine that their results are entirely due to chance alone, and the explanation would appear to be that vaginal PGM is being observed in a significant number of case swabs examined. They emphasized the necessity of knowing the victim's PGM type if PGM typing in vaginal swabs is to be carried out in the investigation of sexual assault cases.

Linde and Molnar (1980) reported on a procedure in which they could simultaneously determine the PGM_1 types of seminal samples from sexual assault cases, and separate the seminal and vaginal acid phosphatases for identification purposes (section 10.3.5).

Renninger and Sina (1970) first noted minor differences in banding patterns between the red cell PGM_1 as against that in spermatozoa. There is a band in approximately the d–e position which is not seen in red cells. The presence of this band was noted by Brinkmann and Koops (1971), and called "d'''". They observed two additional bands, called e′ and f′, running slightly faster than e and f, the latter being characteristic of PGM_1 2 types. They noted that the PGM_3 isoenzymes were present as well. These observations were confirmed and extended by Blake (1976) and Blake and Sensabaugh (1976). They noted that the "extra" band in sperm cell PGM may be the same, but slightly differently expressed in PGM_1 1 and PGM_1 2. The PGM_2 locus was said to be monomorphic in seminal plasma and sperm cells, i.e., the genetic variants seen in the red cells are not expressed. PGM_3 isoenzymes are present and can be grouped, but the concentration is low, and the PGM_1 enzymes have to be overstained to be able to read the third locus types. The differences in PGM_1 expression particularly must be appreciated in reading PGM types from seminal stains or from

semen-positive vaginal swabs. The differences in PGM_1 locus expression, Blake and Sensabaugh said, could account for some of the aberrant results reported by Rees and Rothwell (1975). Sensabaugh *et al.* (1979 and 1980) have found that seminal PGM_1 patterns are altered if the seminal samples are contaminated with saliva. These alterations could lead to typing errors if the presence of the contaminant was unrecognized. Blake (1976) did not find PGM_1 activity in vaginal secretions in the absence of blood, except in one case where the pattern in no way resembled that of human isoenzymes, and was attributed to microorganisms.

Blake and Sensabaugh (1978) have carried out an extensive series of studies on the concentrations of isoenzyme and serum protein (see Unit VII) genetic markers in human spermatozoa and seminal plasma as compared with that in the blood. Most of the PGM activity of semen is attributable to seminal plasma rather than to cells. Semen contains about half the PGM activity found in blood on a per mℓ basis. It was estimated that the minimum quantity of whole semen or seminal plasma needed for PGM detectability was about 1 μℓ.

Sutton (1979a and 1979b) established that the extended *PGM*, system phenotypes detected by isoelectric focusing, can be determined in semen, and further that the seminal type always matched the blood type from the same individual. It was also possible to diagnose the multiple phenotypes in human buccal cells (Sutton, 1979c).

The stability of PGM enzymes has been discussed above in connection with the phenotyping of this system in blood, bloodstains, semen, seminal stains, and vaginal swabs. Rothwell and Sayce (1974) found that PGM isoenzymes in a number of other human tissues, including adipose tissue, muscle, liver and brain, deteriorate very rapidly unless the material is kept at refrigerator or freezer temperatures.

PGM isoenzymes may be determined in the tissues of human teeth, particularly in dental pulp (Suyama and Imai, 1975; Turowska and Trela, 1977). PGM_1 can be phenotyped in hair roots as well (Oya *et al.*, 1978; Twibell and Whitehead, 1978; Yoshida *et al.*, 1979). The isoelectric focusing procedure is equally applicable to hair roots, and the ten phenotypes can be distinguished with this procedure (Burgess *et al.*, 1979).

Brinkmann (1971) gave a good review of the literature of PGM isoenzymes and the medicolegal applications of the system.

27.5 The Distribution of PGM Phenotypes in U.S. Populations

The data are presented in Table 27.1.

Table 27.1 Distribution of *PGM*, Phenotypes in U.S. Populations

Population	Total	Frequency -- Number (Percent) PGM, 1	PGM, 2–1	PGM, 2	Other	*PGM*^{1} ★	Reference
CAUCASIAN							
San Francisco, CA	271	169 (62.4)	83 (30.6)	19 (7.0)		0.777	Lie-Injo, 1966
Chicago, IL	101	68 (67.3)	30 (29.7)	3 (3.0)		0.824	Shih and Hsia, 1969
Seattle, WA	508					0.752	Giblett, 1969
New York, NY	164	102 (62.2)	51 (31.1)	11 (6.7)		0.777	Mondovano and Gaensslen, 1975
Philadelphia, PA	180	(55.9)	(35.1)	(7.9)	(1.1)	–	Polesky et al., 1976
Pittsburgh, PA	1,253	698 (55.7)	487 (38.9)	67 (5.3)	1 PGM, 6–2	0.751	Hagins et al., 1978
California	5,972	(58.9)	(35.6)	(5.4)	(0.1)	0.768	Grunbaum et al., 1978b
Bexar County, TX	200	(64.0)	(30.0)	(6.0)		0.790	Ganaway and Lux, 1978
Detroit, MI	503	288 (57.3)	179 (35.6)	36 (7.2)		0.751	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade Co., FL	367	218 (59.4)	123 (33.5)	26 (7.0)		0.762	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	386	220 (56.9)	149 (38.6)	17 (4.4)		0.763	Siglar, 1979 and see Shaler, (1978)
NEGRO							
San Francisco, CA	284	188 (66.2)	77 (27.1)	17 (6.7)	2 PGM_2 2–1	0.801	Lie-Injo, 1966
Ann Arbor, MI	202	144 (71.3)	52 (25.7)	6 (3.0)	1 PGM_2 2–1	0.84	Brewer et al., 1967a
Chicago, IL	101	62 (61.4)	31 (31.0)	8 (7.9)		0.77	Shih and Hsia, 1969
Seattle, WA	654				4 PGM_2 2–1, 1 PGM_2 3–1	0.809	Giblett, 1969
New York, NY	133	88 (66.2)	39 (29.3)	6 (4.5)		0.808	Mondovano and Gaensslen, 1975
Philadelphia, Pa	180	(59.1)	(35.1)	(3.9)	(1.9)	–	Polesky et al., 1976
Pittsburgh, PA	714	481 (67.4)	209 (29.3)	(3.4)		0.821	Hagins et al., 1978

Table 27.1 (Cont'd.)

Population	Total	Frequency — Number (Percent) PGM, 1	PGM, 2–1	PGM, 2	Other	PGM_1^1 ★	Reference
California	1,024	(66.2)	(29.5)	(4.0)	(0.3)	0.812	Grunbaum et al., 1978b
Bexar County, TX	200	(64.0)	(32.0)	(4.0)		0.800	Ganaway and Lux, 1978
Detroit, MI	504	310 (61.5)	176 (34.9)	18 (3.6)		0.790	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade Co., FL	344	215 (62.5)	113 (32.8)	16 (4.7)		0.789	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	171	118 (69.0)	46 (26.9)	7 (4.1)	1 PGM, 3–2	0.825	Siglar, 1979 and see Shaler, (1978)
CHINESE							
San Francisco, CA	110	64 (58.2)	36 (32.7)	6 (5.5)	3 PGM, 6–1, 1 PGM, 7–1	0.764	Lie-Injo et al., 1968
Seattle, WA	212					0.776	Giblett, 1969
New York, NY	156	98 (62.8)	51 (32.7)	7 (4.5)		0.792	Mondovano and Gaenssien, 1975
ASIAN							
California and Hawaii	3,044	(59.0)	(35.0)	(5.6)	(0.4)	0.769	Grunbaum et al., 1978b
HISPANIC							
New York, NY	129 ●	74 (57.4)	43 (33.3)	11 (8.5)	1 probable PGM, 6^{JAP}–1	0.7461	Mondovano and Gaenssien, 1975
California ☆	1,586	(58.7)	(34.7)	(6.2)	(0.4)	0.764	Grunbaum et al., 1978b
Bexar County, TX	200	(61.0)	(34.0)	(4.0)		0.780	Ganaway and Lux, 1978
Miami/Dade Co., FL	362	204 (56.4)	139 (38.4)	19 (5.2)		0.756	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	198 ◇	117 (59.1)	76 (38.4)	5 (2.5)		0.783	Siglar, 1979 and see Shaler, (1978)

★ Gene frequency ☆ "Chicano/Amerindian" ● Primarily Puerto Rican ◇ Primarily Mexican

SECTION 28. ADENYLATE KINASE

28.1 Recognition of Adenylate Kinase

Adenylate Kinase (AK; ATP:AMP phosphotransferase; E.C. 2.7.4.3; myokinase) catalyzes the reversible conversion of ATP and AMP to ADP, according to the stoichiometry ATP + AMP $\rightleftarrows$ 2 ADP. In 1943, Colowick and Kalckar were studying transphosphorylation reactions in various tissues, and noticed that hexoses could in some way become phosphorylated, apparently enzymatically, in the presence of ADP. The hexokinase reaction, in which ATP transfers its terminal phosphate group to hexose, was well known. At the suggestion of Dr. Marvin Johnson of the University of Wisconsin, they pursued a search for an enzyme that could interconvert ADP and ATP. The enzyme was soon found (Colowick and Kalckar, 1943; Kalckar, 1943), and at the time, it was called myokinase. The reaction was characterized as a "phosphate dismutation" since ADP could be both donor and acceptor of phosphate. In 1951, Colowick re-named the enzyme "adenylate kinase". The presence of the enzyme in red blood cells was noted by Kotel'nikova (1949). In 1958, Kashket and Denstedt in Canada, and Tatibana *et al.* in Japan, recognized the enzyme in human red blood cell lysates. The enzyme was remarkably stable, as noted by a number of these workers. Cerletti and Bucci (1960) looked at the red cell enzyme's properties, and found that it was Mg^{++} activated and optimally active at pH 7.5. It was found in the soluble material of the red cell, and did not appear to be bound to stroma.

28.2 AK Polymorphism

28.2.1 AK_1

In 1966, Fildes and Harris found that red cell adenylate kinase from different persons exhibited electrophoretic heterogeneity on starch gels. Lysates were run in a gel made up in 5 mM histidine, pH 7, and with 0.41M citric acid adjusted to pH 7 with NaOH as bridge buffer. Three phenotypes could be distinguished, and these could be accounted for on the basis of two alleles, AK^1 and AK^2, operating at an autosomal locus. Family studies on 54 matings with 136 children were consistent with the postulated mode of inheritance. In almost 1,000 unrelated English people, some 90% were AK 1, about 10% were AK 2-1, and AK 2 was quite rare. Two different activity detection systems could be used for AK. The reaction sequences for these are shown in Figure 28.1. In the first, ADP is furnished as substrate, and the ATP produced coupled to the Glc-6-phosphate dehydrogenase reaction through the hexokinase reaction to produce NADPH, which can reduce MTT tetrazolium. In the second, an equimolar mixture of ATP and AMP is provided, and the ADP produced allowed to react with PEP to form pyruvate (and ATP). The pyruvate is reduced to lactate by exogenously added LDH, the NADH cofactor which is fluorescent being oxidized to nonfluorescent NAD in the process. Bands of AK activity thus show up as dark zones on a fluorescent background. The first procedure is somewhat more satisfactory and is usually used for routine phenotyping.

The two allele hypothesis of inheritance has been widely confirmed by family studies (Rapley *et al.*, 1967; Bowman *et al.*, 1967; Berg, 1969; Lamm, 1971b; and others). Bowman *et al.* (1967) found another phenotype which was believed to reflect heterozygosity of AK^1 and a new allele, called AK^3. Another phenotype, believed to be the result of a fourth allele, was observed too, and the phenotype was tentatively called AK 4-1. Electrophoresis was carried out by these workers in starch gels using 0.5M phosphate buffer, pH 6.2, for the electrode vessels, and 1:5 dilutions of this buffer for the gel. AK 4-1 was reported in a French family by Rapley *et al.* (1967), and shown to be due to a fourth allele AK^4. Giblett observed another example of an AK 4-1 (personal communication to Harris *et al.*, 1968). In 1972, Santachiara Benerecetti and collaborators found a new phenotype among 600 subjects in southern India, and it was shown to be due to the presence of a fifth allele, AK^5. The phenotype

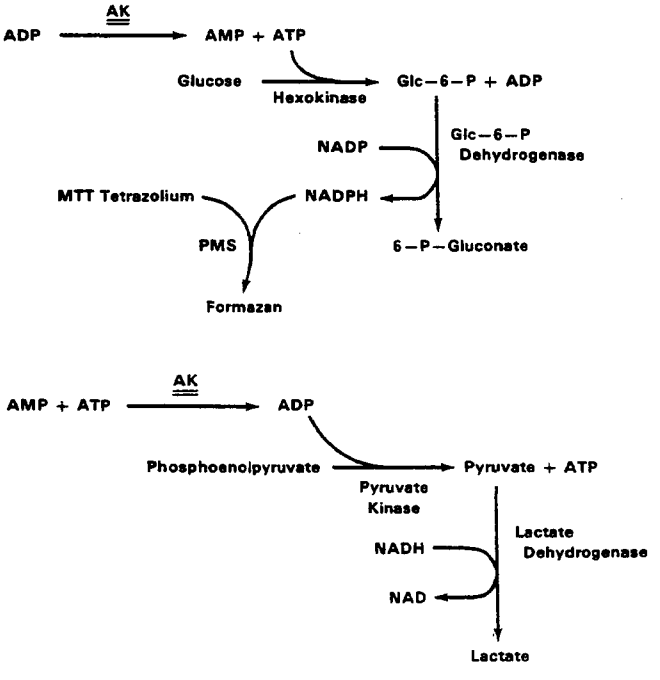


Figure 28.1 Detection Reaction Sequences for AK

observed was an AK 5-1 (Santachiara Benerecetti *et al.*, 1972a). The patterns for the AK phenotypes seen if electrophoresis is carried out at pH 7 are indicated diagrammatically in Figure 28.2.

There are a few reports of families having members lacking AK activity. Szeinberg *et al.* (1969a and 1969b) found an Arab boy and his sister who both had very low AK activity. The parents, who were related, and four other sibs, had AK activity varying from about 22% to 72% of normal. Some of the people had anemia, and the AK deficiency appeared to be transmitted autosomally, with partial expression in the heterozygotes. Singer and Brock (1971) found a patient with 50% normal activity, who was an AK 1. The half-normal activity was segregating in the patient's family, and was most easily accounted for by the presence of a silent allele. Weissmann and Pribeen (1979) formed an apparent AK° in a family in which two AK 2 children had been born to an AK 1 mother where the father was AK 2-1.

28.2.2 Additional AK loci—linkage relations

In 1970, Brock looked at the AK isozymes in a variety of human tissues (Brock, 1970a). Three sets of isozymes could be distinguished in the varous tissues, and were not expressed in the same way in different ones of them. The suggestion, however, was that the differences could be accounted for by differential expression of similar genotypes, and by the fact that there was complexation of hemoglobin by red cell AK. In 1972, Khoo and Russell found that the AK isozymes of both human and rabbit tissues could be distinguished not only on the basis of electrophoretic mobility, but also by differential inhibition of the enzymes with $AgNO_3$ and anti-rabbit muscle AK serum. In both species, the isozymes of the red cell, skeletal muscle and brain were similar, and inhibited by low concentrations of silver ion, while those of liver, kidney, spleen and heart were similar, and relatively insensitive to silver ion inhibition. Nguyen *et al.* (1972) thought that the "second set" of isozymes was the result of an additional AK locus, AK_2, and man-mouse hybrid cell studies indicated that AK_2 was syntenic with PGM_1 and Peptidase C loci on chromosome 1. In 1974, Russell *et al.* studied the AK isoenzymes of a number of human tissues, and said that on the basis of a number of different properties, there were at least six different tissue-specific AK isozymes. Some of these differed in MW as estimated by gel filtration chromatography.

In 1976, Wilson *et al.* found a set of isoenzymes active with GTP or ITP and AMP, but not with ATP and AMP. These enzymes were resistant to inhibition by Ag^+. Actually, this activity corresponds to the enzyme called GTP: AMP phosphotransferase (E.C. 2.7.4.10), but it was attributed to a third locus of AK, called AK_3. No genetic variation has yet been reported at AK_2 or AK_3, and the products of these loci are not expressed in red cells nor in skeletal muscle tissue. AK_1 has been assigned to chromosome 9, linked to the ABO and nail-patella syndrome loci (Westerveld *et al.*, 1970). Povey *et al.* (1976) confirmed the AK_1 assignment, and said that AK_3 was also assigned to chromosome 9, syntenic with the soluble aconitase locus.

28.3 Biochemical Studies on AK

The early studies by Kalckar and Colowick were mentioned in section 28.1. The older biochemical studies have

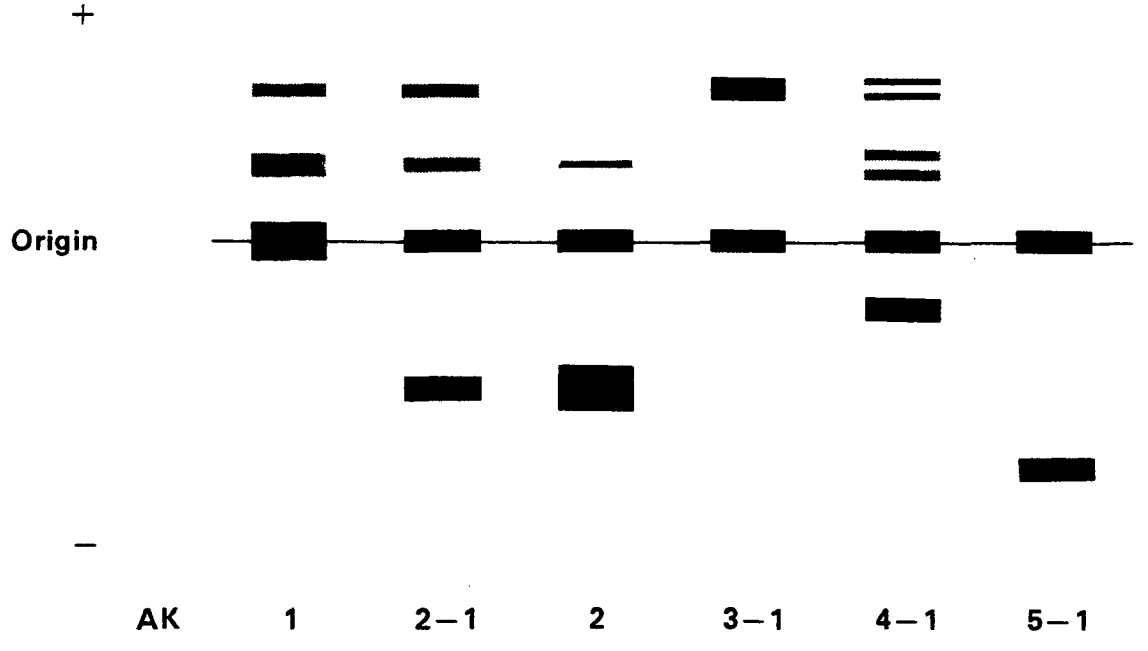


Figure 28.2 Electrophoretic Patterns of AK Phenotypes at pH 7.

been reviewed by Noda (1962), and the more recent work by the same author (Noda, 1973). The enzyme from muscle has 2 sulfhydryl groups per molecule, and although −SH reagents inhibit enzymatic activity, the −SH groups do not appear to be directly involved in substrate binding, nor in catalysis *per se*. There is good evidence that a histidine residue is involved in the catalytic mechanism. A divalent metal ion is required for AK activity, and Mg^{++} is the best one for the AK from most sources. In some cases, Ca^{++}, Mn^{++}, Co^{++} or Ba^{++} can substitute for Mg^{++}, but activity is lower. The $Mg^{++}$is required to bind the substrate, and the reaction can be written as: MgATP + AMP ⇄ MgADP + ADP. AK may be assayed in solution by coupling the ATP produced to the pyruvate kinase reaction, and then to the LDH reaction, and measuring the decrease in absorbancy at 340 nm (Levin and Beutler, 1967). This scheme may also be used for the detection of AK isozymes in gels (Figure 28.1).

AK from human muscle has been extensively purified and crystallized (Thuma *et al.*, 1972). The K_m for all three nucleotides was found to be about 0.3 mM, and the MW was estimated as 21,500 by sedimentation, and as 21,700 by calculation from the amino acid composition. These values are very similar to those reported for other mammalian muscle AK enzymes. Harris *et al.* (1968) estimated the MW of the red cell enzyme as 24,000 by gel filtration chromatography, in complete agreement with the estimate of Bowman *et al.* (1967) by the same technique. There are indications that the enzymes from different human tissues may differ in MW in some cases (Russell *et al.*, 1974)

Rapley and Harris (1970) found that the mean activity of lysates from AK 1 adults was significantly higher than the activity of those from AK 2-1 adults. The differences are not seen in lysates from newborns. At the same time, newborn lysates show only about 65% of the total AK activity seen in adult lysates.

There was a suggestion in the literature by Bockelmann *et al.* (1968) that pyruvic kinase (PK; E.C. 2.7.1.40) and AK share a subunit. The suggestion was based on evidence obtained in detecting the enzymes after electrophoresis. The detection reaction sequences overlap, as may be readily appreciated by looking at Figure 28.1. PK does not show genetic variation. However, Brock (1970b) said that the assay system could be adjusted so that AK is selectively inhibited while PK is detected, and that his results gave no indication of a common subunit.

28.4 Medicolegal Applications

28.4.1 Disputed parentage

AK phenotyping can be usefully applied to cases of disputed parentage, although the probability of excluding a falsely accused man by the AK system alone is not very high. Prokop and Göhler (1976) said that only 3.5 to 4% of true nonfathers would be excluded by AK alone in middle European populations. In England, the figure is about 4.5% (Boorman *et al.*, 1977). Polesky *et al.* (1976), said that the figure was 4.29% for Caucasians, and only 0.66% for Black people. Possible occurrence of the rare silent allele, AK_1^0 should be kept in mind in the interpretation of exclusions of the second order based on AK.

Chen *et al.* (1977) have shown that the AK isoenzymes are fully developed in fetal blood.

28.4.2 AK phenotyping in bloodstains

In 1968, Culliford and Wraxall reported that AK phenotypes could be reliably detected in bloodstains by starch gel electrophoresis. The original procedure of Fildes and Harris (1966) was first successfully employed, but it was soon found that the thin starch gel method (Wraxall and Culliford, 1968) was more satisfactory for small amounts of bloodstained material. The pH 7 system of Fildes and Harris (1966) was used. The results of additional investigations on phenotyping the enzyme in bloodstains were given by Culliford (1971). Phosphate reaction buffers should be avoided with AK since they were found to inhibit the enzyme. Absolute activity values were not given, but if the enzyme is taken to have full activity at 0.01M phosphate at pH 6.8, then 80 mM phosphate inhibited about 50% and 0.2M phosphate almost completely. The AK 2 isozymes appeared to be more sensitive to this inhibition than the AK 1 isozymes. Succinate was found to have an activating effect on AK, and a pH 5 buffer system consisting of Tris-succinate for the gel, and citric acid-NaOH for the electrode vessels was successfully employed.

Other electrophoretic media than starch have been employed for AK, as have a number of different buffer systems, although not all of these have necessarily been tested in the phenotyping of dried bloodstain material. Skude and Jakobsson (1970) used 1% agar gels prepared from Reinagar for electrophoresis, with 50 mM phosphate, pH 6.2, buffers. Thirty lysates could be run on a plate about 10 × 20 cm in about 45 minutes. Thin-layer agarose electrophoresis was employed by Tsuji and Weissman (1977). Hoppe *et al.* (1972) gave a procedure for phenotyping AK in lysates on horizontal polyacrylamide gels.

Rosalki (1970) reported successful typing of AK on cellulose acetate membranes, using either the Beckman Microzone system or the Gelman Sepraphore III system. Sonneborn and Renninger (1971) confirmed these results using different buffers. Sonneborn (1972) discussed the subject further in connection with a number of isoenzymes. Saenger and Yates (1975) applied a system similar to that of Sonneborn and Renninger (1971) to AK phenotyping in bloodstains. Phosphate buffer, pH 6.25, in a final concentration of about 1.55 mM was used, the electrophoresis being completed in about 70–90 minutes. Stombaugh and Kearney (1977) found the Beckman Microzone system applicable to bloodstain phenotyping, and sixteen samples could be typed simultaneously.

There are a number of procedures reported in which AK is simultaneously determined with other isoenzymes in the same electrophoretic operation: AK with ADA (Kirchberg and Wendt, 1970), AK with PGD (Brinkmann and Thoma, 1970), AK with ADA (Hummel, 1970), AK with ACP, PGM, and ADA (Martin and Niebuhr, 1971), AK with

ADA and PGD (Brinkmann and Dirks, 1971), AK with ADA and PGM on acrylamide (Wrede *et al.*, 1971), AK with GPT, PGD and PGM (Goedde and Benkmann, 1972), AK with PGM and PEPA (Neilson *et al.*, 1976) and AK with ACP and ADA (Wraxall and Stolorow, 1978). An effort to combine AK with PGD on a single plate for bloodstain grouping was made by Culliford (1970). Phosphate buffers were used, but the system was considered unsatisfactory because low activity stains from AK 2-1 people could give apparent weak AK 1 results. The Wraxall and Stolorow (1978) procedure was specifically devised for dried bloodstain phenotyping.

28.4.3 Survival of AK in blood and bloodstains

AK is one of the more stable enzymes. In the original bloodstain phenotyping work, it was noted that AK had been typed in stains up to 3 months old (Culliford and Wraxall, 1968). Culliford (1971) said that stains up to 6 months old had been typed, but that losses of activity in bloodstains after 3–4 weeks aging could occur commonly. He said further that the succinate buffer system (pH 5) was to be preferred over the histidine (pH 7) (section 28.4.1) since, in the latter, the 2 band weakened in older stains and the 1 band tended to become diffuse. Brinkmann and Dirks (1971) said that AK could be detected in stains up to 11 months old. Rothwell (1970) found that AK could sometimes be typed in stains up to 11 months old. In a group of bloodstains, about 2/3 were typable at 5 months aging, and about 15% after six months. Some stains were untypable after 2 months, however. Welch (1972b) found that AK was completely typable during the entire course of a 30 day aging study. Saenger and Yates (1975) found that AK could be typed in stains up to 6 months old, after which the limiting factor seemed to be the inextractability of the enzyme from the older stains. Stombaugh and Kearney (1977) found that bloodstains could be typed for up to 3 months if stored at 37°, while those kept at 4° or frozen could be typed up to 18 months. Denault *et al.* (1978) found that AK 1 bloodstains on a variety of substrata were typable up to 26 weeks, regardless of whether they had been stored at high or low humidity.

AK in lysates is stable for lengthy periods of time if the samples are kept frozen (Rothwell, 1970; Culliford, 1971), and for years if kept under liquid nitrogen. In samples collected for blood alcohol determinations, and kept at room temperature, Rees *et al.* (1975) found that AK determinations were reliable for up to 100 days. At 109 and 122 days of aging, AK 2-1 samples were read as AK 1, while AK 1 samples were read correctly. As noted previously for bloodstains by Culliford (1971), therefore, the 2 isoenzyme appears to be more labile than the 1 isoenzyme.

28.4.4 AK phenotyping in other tissues

AK isozymes of the AK_1 polymorphic locus may be detected in skeletal muscle and some other tissues without undue difficulty. Rothwell and Sayce (1974) found that deterioration of activity in tissues is very rapid unless the specimens are kept cold or frozen, in which case the samples were stable for months. Oepen (1974) found that AK typing from skeletal muscle was fully reliable in terms of being identical to the red blood cell type. Turowska and Trela (1977) reported phenotyping of AK from dental pulp.

AK is present in spermatozoa, but there is very little activity in seminal plasma (Blake, 1976; Blake and Sensabaugh, 1976 and 1978). Expressed in terms of Units/mℓ, seminal plasma has about 1/1,000 the AK activity of blood. A unit of AK activity was defined as 1 μmol NADP reduced/min at 37° and pH 7.5 in a hexokinase-G6PD coupled assay system. The AK 2 isoenzyme is extremely labile in sperm cells, 24 hours storage of a specimen at 4° being sufficient to reduce its activity enormously. For this reason, it cannot be regarded as a good genetic marker in spermatozoa.

28.5 Distribution of AK Phenotypes in U.S. Populations

The data are shown in Table 28.1. AK, though very stable, is not an especially good marker in populations, the probability of distinguishing between two random people on the basis of AK being about 18%. It is of interest to note that the AK^2 gene is virtually absent in Chinese, the sole AK 2-1 individual in all the literature having been reported by Shih *et al.* (1968a). World data for AK were given by Tills *et al.* (1970a), and a 10-fold error in the figures given in that paper was corrected in Tills *et al.* (1971a).

Table 28.1 Distribution of *AK* Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) AK 1	AK 2–1	AK 2	*AK'*★	Reference
CAUCASIAN						
Chicago, IL	1,315 ☆	1,193 (90.7)	118 (9.0)	3 (0.3)	0.9525	Bowman et al., 1967
Ann Arbor, MI	254	240 (94.5)	14 (5.5)	0	0.9724	Brewer et al., 1967
Seattle, WA	172	163 (94.8)	9 (5.2)	0	0.969	Giblett, 1969
New York, NY		127 (93.4)	9 (6.6)	0	0.9669	Mondovano and Gaensslen, 1975
Philadelphia, PA	180	(97.3)	(2.7)	(0)	–	Polesky et al., 1976
Washington, DC	364	338 (92.9)	25 (6.9)	1 (0.2)	0.9629	Stombaugh and Kearney, 1977
California	5,969 ⭘	(92.7)	(7.1)	(0.1)	0.963	Grunbaum et al., 1978b
Detroit, MI	503	474 (94.2)	29 (5.8)	0	0.971	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade County, FL	366	339 (92.6)	26 (7.1)	1 (0.3)	0.962	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	115	108 (93.9)	7 (6.1)	0	0.970	Siglar, 1979 and see Shaler, (1978)
NEGRO						
Chicago, IL	1,063 ☆	1,049 (98.7)	13 (1.3)	0	0.9934	Bowman et al., 1967
Ann Arbor, MI	139	135 (97.1)	4 (2.9)	0	0.9856	Brewer et al., 1967a
Seattle, WA	223	220 (98.7)	3 (1.3)	0	0.993	Giblett, 1969
Chicago, IL	101	99 (98.0)	2 (2.0)	0	0.991	Shih and Hsia, 1969
New York, NY	134	130 (97.0)	3 (2.2)	1	0.981	Mondovano and Gaensslen, 1975
Philadelphia, PA	180	(100)	(0)	(0)	1.000	Polesky et al., 1976
Washington, DC	76	75 (98.7)	1 (1.3)	0 (0.8)	0.9934	Stombaugh and Kearney, 1977
California	965	(98.4)	(1.6)	(0)	0.992	Grunbaum et al., 1978b
Detroit, MI	504	501 (99.4)	3 (0.6)	0	0.997	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade County, FL	346	339 (98.0)	7 (2.0)	0	0.990	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	54	53 (98.2)	1 (1.8)	0	0.991	Siglar, 1979 and see Shaler, (1978)
HISPANIC						
New York, NY	136 ▢	130 (95.6)	6 (4.4)	0	0.978	Mondovano and Gaensslen, 1975
California ●	1,344	(95.6)	(4.3)	(0.1)	0.978	Grunbaum et al., 1978b
Miami/Dade Co., FL	357	339 (95.6)	18 (4.4)	0	0.975	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	74 ■	69 (93.2)	5 (6.8)	0	0.966	Siglar, 1979 and see Shaler, (1978)
CHINESE						
New York, NY	156	156 (100)	0	0	1.000	Mondovano and Gaensslen, 1975
ASIAN						
Seattle, WA ◇	146	146 (100)	0	0	1.000	Giblett, 1969
California and Hawaii	2,304	(99.8)	(0.2)	(0)	0.999	Grunbaum et al., 1978b

★ Gene frequency ☆ One person was AK 3–1 ⭘ 0.1% were rarer phenotypes ● "Chicano-Amerindians" ◇ "Mixed Oriental" ▢ Primarily Puerto Rican ■ Primarily Mexican

SECTION 29. ERYTHROCYTE ACID PHOSPHATASE

29.1 Recognition of Acid Phosphatase in Blood

Erythrocyte acid phosphatase (ACP; EAP; E.C. 3.1.3.2) is systematically known as orthophosphoric monoester phosphohydrolase. The name of the enzyme is abbreviated as "ACP" in this book, following Harris and Hopkinson (1976), for the reasons noted in section 29.3.1.

In 1924, it was noticed independently in two different laboratories that drawn blood, left standing for a time, showed an increase in its inorganic phosphate content (Lawaczek, 1924; Martland and Robinson, 1924). The phosphate level increased more rapidly if the blood was left at 37° than at room temperature. It was correctly recognized by Martland *et al.* (1924) that this inicrease was the reflection of an enzymatic activity present in red cells, for the effect was pronounced only when the cells were lysed. The enzyme was called "phosphoric esterase", and could be shown to hydrolyze hexose phosphate, hexose diphosphate and glycerophosphates. Roche (1931) confirmed these findings. The red cell enzyme had a pH optimum of 5.8 with p-nitrophenyl phosphate, and hydrolyzed the α-isomer of glycerophosphate better than the β-isomer. Woodard (1942) noted the presence of "acid" phosphatase activity in a number of tissues. Earlier studies on the catalytic properties of red cell acid phosphatase were carried out by Tsuboi and Hudson (1953, 1954 and 1956).

29.2 ACP Polymorphism

Hopkinson *et al.* (1963) first reported genetic variation of ACP. Five phenotypes were seen by starch gel electrophoresis, using gels made in Tris-succinate, pH 6, with citric acid-NaOH, pH 6, bridge buffer. The zones of enzyme activity were best detected with 5mM phenolphthalein-diphosphate in a citrate buffer, at pH 6, followed by incubation with ammonia to make the gel basic, and the phenolthalein colored. More diffuse bands were said to be seen if p-nitrophenyl phosphate was used as substrate, and the enzyme showed no activity with naphthyl phosphates (but see section 29.3.2). Formalin inhibited activity, but tartrate did not. Acid phosphatase assay (detection) techniques with a variety of substrates were discussed in section 10.3.4, and the material in that section is equally applicable to acid phosphatase enzymes, regardless of the source.

Family studies were consistent with a genetic explanation for ACP polymorphism based on three allelic genes, called P^A, P^B, and P^C, determining 6 phenotypes called A, BA, B, CA, CB and C. Gene frequencies in 139 English people were about 0.35 for P^A, 0.6 for P^B and 0.05 for P^C. Homozygous ACP–C persons were to be expected only about once in 400 persons in this population. Additional family and population studies (Hopkinson *et al.*, 1964) confirmed the genetic hypothesis. Many other family studies (e.g. Prokop, 1967) have been in complete accord. In 1964, Lai *et al.* found an ACP–C individual in a Brazilian population, and studies on 80 families fully confirmed the three allele scheme of inheritance for the system. In 1965, Giblett and Scott found a new phenotype in a Black person in Seattle. They carried out electrophoresis according to the original method of Hopkinson *et al.* (1963), but with formic acid-NaOH, pH 5, buffers as well. The latter were best for detecting the new phenotype, which was believed to be the result of the heterozygosity of P^a with a new allele P^r. The phenotype was called RA. The symbols P^a and P^r are equivalent to the symbols P^A and P^R. Another phenotype was reported by Karp and Sutton (1967) which was the result of another allele, P^D, at the ACP locus in heterozygous combination with P^B. This BD phenotype was seen in two Black males. Karp and Sutton used citrate-phosphate buffers, pH 5.9, as well as phosphate, pH 6.2, buffers in their studies. Further examples of the RA phenotype of Giblett and Scott (1965) were seen, as well as several RB people and one RC. The P^R gene appeared to be restricted to Black populations. It has not been reported in Caucasians, but it has been seen in Chinese. Shih and Hsia (1969) found 2 RB in 100 Chinese from Taiwan whom they grouped for ACP. This fact is not mentioned in the original paper, but was ascertained from a personal communication to Mourant *et al.* (1976). P^R occurs more commonly in some southern African Black populations, particularly in the people called Khoisan, with a gene frequency in the neighborhood of 0.2 to 0.25 (Jenkins and Corfield, 1972). The results of Shih and Hsia suggest that P^R may have an appreciable frequency in Chinese, or perhaps that the selection of persons for grouping in this case was accompanied by a bit of luck. The P^D allele was found in a European by Lamm (1970b). One BD was seen in 209 unrelated Scandinavian adults. Lamm's family studies (1970b) were fully in accord with the three allele pattern of inheritance as well. A further allele, P^E, was found in a 55 year old Danish man. He was BE, and his only living near relative, a daughter, had not inherited it from him (Sørensen, 1975).

Silent alleles have been observed at the *ACP* locus. In 1969, Herbich encountered an apparently silent allele of ACP segregating in three generations. A BA mother and an "A" father had a "B" daughter, who, in turn, had a "C" son by a CA father. The best explanation for the observations was heterozygosity for a silent allele, P^o. This conclusion was supported by activity studies which showed that the suspected heterozygotes showed only about half the activity of normal controls. Studies on this interesting Viennese family were extended by Herbich *et al.* (1970). Turowska *et*

al. (1977) reported a silent allele in a south Polish family, this case also being supported by activity measurements. The family came to their attention because of an apparent mother-child incompatibility. A second Polish family with a silent allele segregating in three generations was described by Turowska and Bogusz (1978). Brinkmann *et al.* (1974) reported a number of German families in which P^o appeared to be segregating. They estimated that the allele seemed to occur in about 1 or 2 of every 2,000 people. Nezbeda (1979) found a silent allele of acid phosphatase segregating in three generations of a Czechoslovakian family.

In 1973, Gussmann encountered a peculiar kind of CB phenotype, which was electrophoretically identical to the usual CB, but was very weak. This was called a "BC^x". Smerling (1973) reported that he had encountered three new phenotypes in connection with a case. These were not understandable in terms of the P^R or P^D genes. White *et al.* (1979) described an apparently new phenotype of acid phosphatase in two unrelated samples. The patterns were similar to CA on starch gels in citrate-phosphate buffers (pH 5.5), but the b_2 band was absent.

The ACP system is thus governed by three relatively common alleles P^A, P^B and P^C; there are three rarer alleles, P^R, P^D and P^E, and there can be a rare silent allele P^o in an occasional person.

The ACP phenotypes as seen in citrate (or other tricarboxylic acid) containing buffers are indicated diagramatically in Figure 29.1. The mobility of the A bands is sensitive to buffer composition, and this effect should be appreciated when comparing patterns from different sources. The presence of citrate or other tricarboxylic acids results in what Hopkinson and Harris (1969a) called a "fast A" pattern. A "slow A" pattern is seen in phosphate buffers at about the same pH (near 6). "Intermediate" patterns can be seen with various dicarboxylic acids. Results with citrate bridge buffer and Tris-succinate gel buffer are of the "fast A" category. Further details of this effect have been discussed by Hopkinson and Harris (1969a).

Brinkmann *et al.* (1971) did an interesting analysis of the aggregate ACP phenotypic frequency data in the published literature for something more than 13,000 Europeans. When a X^2 test of significance was done on the data, there was a significant discrepancy between the expected and observed values, almost entirely accounted for by the CB and C frequencies. The simplest interpretation of this finding was that significant errors had been made in reading CB and C phenotypes. Correct phenotyping of ACP should not necessarily be considered simple, therefore, even with hemolysates.

Petersen Inman (1980) did a very similar analysis of the ACP frequency data reported by Grunbaum *et al.* (1978b), and suggested that the lack of goodness of fit between the "expected" and "observed" values might indicate misclassification of some phenotypes. Selvin (1980a) replied to these observations, saying that certain of the ACP phenotypes, particularly the "C" phenotypes, could be difficult to classify in some samples. He noted, however, that the gene frequencies would be changed very little by the level of misclassification that may have been present in the data; Selvin (1980b) has treated the problem of gene frequency estimates in the presence of specific patterns of misclassification in a more general paper as well.

Problems with the phenotyping of ACP, particularly with certain of the "C" phenotypes are discussed in section 29.5.2. There is no doubt that certain phenotypes can be misjudged in this system, and that certain types of samples can cause more problems than others. A modest level of misclassification will not appreciably change the gene frequency estimates in a large population study, as Dr. Selvin (1980b) has shown, and may not, therefore, affect the data obtained very much at all. No level of error or misclassification would be tolerable, however, in the typing of ACP (or any other system) in an individual medico-legal case; here, unlike in a population survey, the individual sample types could greatly influence the outcome of the case.

29.3 Additional Genetic Loci Determining Acid Phosphatase Enzymes—Tissue Acid Phosphatases

29.3.1 Human tissue acid phosphatase isoenzymes

Apart from the red cell ACP, the other medicolegally significant acid phosphatase is the prostatic one, which occurs in relatively high concentrations in most examples of human seminal plasma, and is the basis of the most widely employed non-morphological test for semen identification (section 10.3). The genetic relationships between the ACP of the red cell, the prostate and a variety of other body tissues have only recently become clear. Tissue acid phosphatases, and their relationship to the red cell enzyme, are discussed in this section.

Recognition of the ACP activity in red cells was discussed in section 29.1. Recognition of the prostatic enzyme was discussed in detail in secton 10.3.2. Kutscher (1935) found an acid phosphatase activity in urine, which prompted him to look further for the source of the activity, particularly in male reproductive tract secretions. A high concentration of the enzyme was present in seminal plasma, and was shown to be of prostatic origin (Kutscher and Wohlbergs, 1935; Kutscher and Wörner, 1936). The Gutmans recognized the potential clinical significance of this enzyme when it was noticed that prostatic tumors led to significant increases in the ACP levels in serum and in metastasizing tissue (Gutman *et al.*, 1936; Gutman and Gutman, 1938). This enzyme displays electrophoretic heterogeneity (Sur *et al.*, 1962; Smith and Whitby, 1968; and see in section 10.3.6).

Lundin and Allison (1966a and 1966b) found electrophoretic heterogeneity in the ACP in a variety of human and animal tissues. In 1967, Beckman and Beckman reported the results of their investigations on the ACP from a number of different human organs, and from placental tissues of 1,200 individuals. Four zones of activity could be present, each organ giving a characteristic isoenzyme pattern. The zones were called "A" through "D", "A" being the most anodal

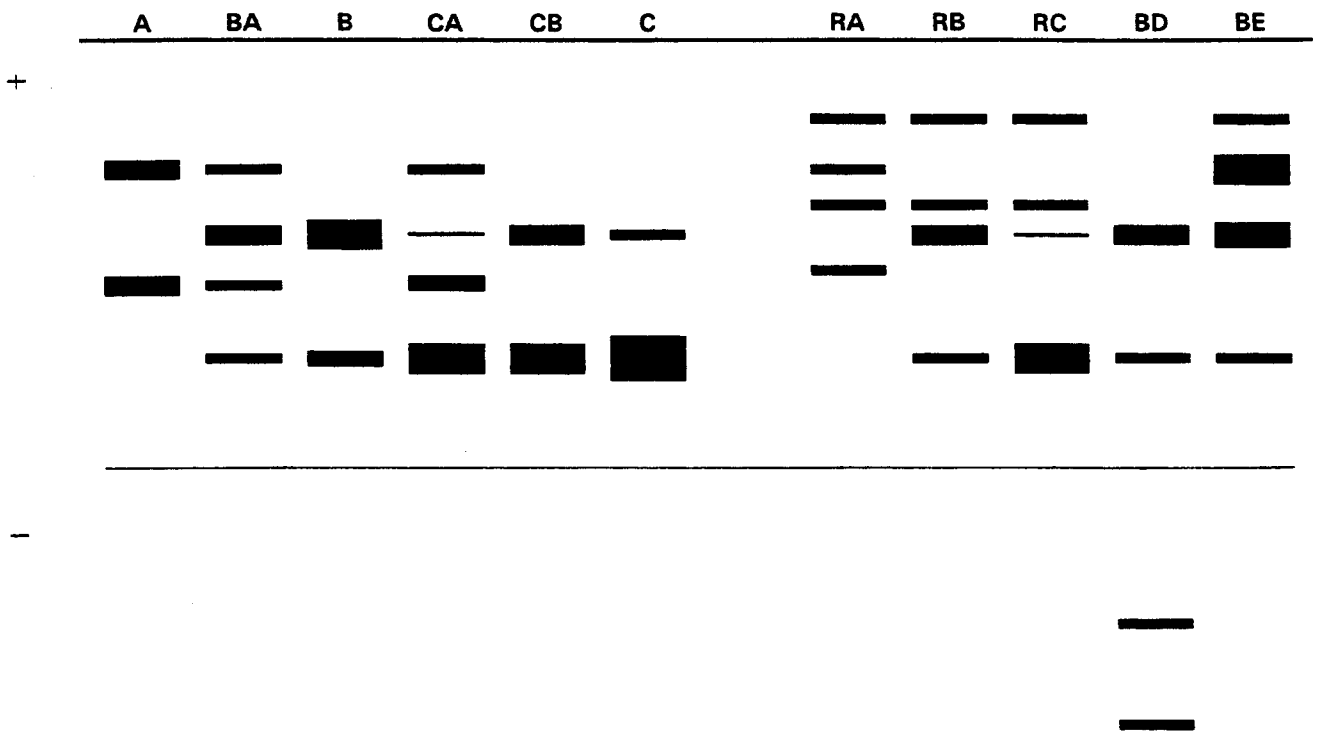


Figure 29.1 Electrophoretic Patterns of ACP_1 Phenotypes in pH 6 Buffers Containing Citrate

at pH 8.6. C zone was found only in placental tissue along with B and D. In three placentae, deviant patterns were seen. All the isozymes were inhibited by L-tartrate and reacted with a specific rabbit antiserum, and differed in pH optima, thermostability and sialic acid content. The four isoenzymes could be found in extracts of cultured human cells as well (Beckman *et al.*, 1968), C occurring regularly in fetal, but not in adult tissues. The variant seen in placental tissue (Beckman and Beckman, 1967) could be reflected in leucocytes which, however, do not have quite the same profile of isoenzymes, and mononuclear cells differ somewhat from polymorphonuclear ones (Beckman *et al.*, 1970b). It was suggested, therefore, that the placental and leucocyte enzymes might have a subunit in common. Others have looked at the ACP isozymes in leucocytes from normal subjects as well as from those with various diseases (Li *et al.*, 1970; Avila and Convit, 1973). In 1972, Swallow and Harris examined the ACP isozymes of 963 placentae and found an additional variant, which was shown to be inherited through family studies using leucocytes. It was found too that the A, B and C isoenzymes had molecular weights of about 95,000, 106,000 and 120,000, respectively, as estimated by gel filtration chromatography. A genetic explanation for the A, B and C isoenzymes, consistent with their own data and with Beckman's data, was proposed in which two independent loci were involved in the synthesis of the isoenzymes. Each locus coded for a different polypeptide chain, and the chains were called α and β. Assuming that the isozymes were dimers, the A, B and C isozymes would then have the compositions $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, respectively. Swallow *et al.* (1973) suggested that the structural loci determining the ACP enzymes be designated "*ACP*". Using methylumbelliferyl phosphate as substrate, it could be shown that the "red cell" enzymes are not restricted to the red cell, but can be identified in other tissues as well. In sum, they said, there are then three distinct *ACP* loci, designated ACP_1, ACP_2 and ACP_3. ACP_1 codes for the "red cell" isoenzymes, and is the only one of the three loci exhibiting genetic polymorphism. The other two loci code for enzymes that can be seen in placenta, white cells and other tissues. Although they are apparently not polymorphic, an occasional rare variant is seen, and it is on this basis that they may be distinguished. ACP_2 codes for the β peptide, and ACP_3 codes for the α peptide, which make up the A, B and C isozymes (Swallow and Harris, 1972). The variant described by Beckman and Beckman (1967) reflects a rare allele of ACP_2, while the variant described by Swallow and Harris (1972) reflects a rare allele of ACP_3. In these terms, it could be said that most people are ACP_2^1 and ACP_3^1 homozygotes. Beckman and Beckman's (1967) variant person could be designated $ACP_2^1ACP_2^2$, while Swallow and Harris' (1972) one would be $ACP_3^1ACP_3^2$. Similarly, the "red cell" locus alleles may

be designated ACP_1^A, ACP_1^B, ACP_1^C, ACP_1^R, ACP_1^D, ACP_1^E and ACP_1^o. This "red cell" acid phosphatase nomenclature is followed in this book, as it appears to reflect the genetic situation more accurately than the older usages.

29.3.2 Some comparisons of the various acid phosphatases

Molecular heterogeneity among acid phosphatases is a generalized biological phenomenon. In human tissues, it appears that there are at least three structural loci determining enzymes with acid phosphatase activity. In any given tissue, the multiplicity of enzymes present is, thus, a reflection of the expression of one or more of these loci. The isoenzymes observed result from differences in primary gene products, as well as from post-synthetic alterations in the primary gene products. The number of distinct activities observed also depends on the substrate used, since the different isoenzymes show varying substrate specificities. The relationships between the various isozymes from all the various different tissues are not yet fully clear.

Two major methods have been used to fractionate the isoenzymes of various tissues: electrophoresis and gel filtration chromatography. Electrophoresis separates the proteins on the basis of net charge primarily, while gel filtration does so on the basis of molecular size. Electrophoretic studies on the placental and white cell ACP enzymes particularly may give different results, depending upon the substrate employed in the detection scheme.

It is now clear that "red cell" ACP isozymes occur in placental tissue, the phenotype corresponding to the red cell type of the fetus. The isozymes are detectable in placental tissue using phenolphthalein diphosphate (Blake *et al.*, 1973a) or methylumbelliferyl phosphate (Swallow *et al.*, 1973) as substrates. Blake *et al.* (1973a) found that there are some differences in the expression of ACP_1 phenotypes in placentae as against in red cells. A minor fast band is seen in placental B and CB phenotypes, which is just slightly faster than the most anodal component of A, BA and CA phenotypes in red cells. In B phenotype samples, the slower band is the more intense in placenta, whereas in red cells, it is the other way around. The CB phenotype shows a more intense slow band in red cells, while in placenta, the intensity of the slow band is proportionately greater. In the original studies, Hopkinson *et al.* (1963) observed no activity with α- or β-naphthyl phosphate. Sørenson (1970) showed, however, that the red cell enzyme does in fact hydrolyze β-naphthyl phosphate, but not the α-isomer. A number of diazonium salts, with which the liberated β-naphthol is coupled to form a colored product (see in sections 10.3.2 and 10.3.4), were found to inhibit the ACP enzymes. If substrate and diazonium salt were added to the reaction mixture together, therefore, no activity was seen. But if substrate was added, and allowed to incubate and react before adding the diazonium salt, clear zones of activity were seen. Fast garnet GBC salt (C.I. 37210) was found to be the best coupling salt of the several tested in the experiments. The β-naphthyl phosphate gave more zones of activity than phenolphthalein disphosphate, and was more sensitive. The ACP isoenzymes were about 10% less active by quantitative assay with β-naphthyl phosphate than with p-nitrophenyl phosphate. Blake *et al.* (1973a) looked at placental tissue ACP using the naphthyl phosphates as substrates. The "red cell" acid phosphatases gave somewhat different patterns in placentae with β-naphthyl phosphate than with phenolphthalein diphosphate, and these were not as easy to identify. Further complicating the picture was the fact that placenta showed ACP activity patterns with α-naphthyl phosphate, which does not react with ACP_1 products. The ACP activity patterns in a number of other tissues were examined in these studies as well.

If MW is used as a basis for dividing the ACP enzymes into categories, certain patterns become apparent, just as is the case when electrophoresis is used. The correlations between the MW classes and the electrophoretic ones are not usually apparent. A very good over-all view of the ACP isoenzymes considered from the point of view of MW is given by Sensabaugh (1975). Generally, there are four MW classes of ACP enzymes in mammalian (including human) tissues. These may be partitioned by gel filtration chromatography, as has been done with bovine kidney and liver tissues (Heinrikson, 1969), human placenta (DiPietro and Zengerle, 1967) and a number of other human tissues (Sensabaugh, 1975). Sensabaugh (1975) found that a high MW enzyme (on the order of 200,000) was present in most tissues, but not in red cells nor in semen. A second class (MW 100,000 to 130,000) was present in all tissues except for red cells. Another class had MW in the range of 30,000 to 60,000, and is not present in all tissues. The fourth class is present in all tissues except semen and prostatic tissue extracts. This class is of low MW (about 13,000–20,000), includes the erythrocyte ACP enzymes, and is perhaps the best characterized of the four. These classes of enzymes show some other properties in common within a given class, apart from being of similar MW. The low MW enzymes, including the red cell ones, are inhibited by formaldehyde (Abul-Fadl and King, 1948) but not by tartrate, which strongly inhibits the prostatic (Abul-Fadl and King, 1949) and other high MW phosphatases. The 30,000–60,000 MW class is activated by Mg^{++}. The red cell enzymes show a preference for FMN (riboflavin-5′-phosphate) as substrate (Luffman and Harris, 1967; Sensabaugh, 1975), a property shared by the low MW (16,300–16,600) enzyme purified from beef liver by Heinrikson (1969). And the red cell enzyme is activated by L-methyl adenine (Sensabaugh, 1975), a property shared by the low MW enzyme purified from human placenta (DiPietro and Zengerle, 1967). The placental enzyme hydrolyzes 17-β-estradiol-3-phosphate (DiPietro, 1968). The low MW enzymes, generally, show significant activity with a restricted number of phosphate esters. Sensabaugh (1975) presented some correlations between the electrophoretically distinct ACP's and the MW categories. The highest MW fraction appeared to be associated with the "A" band of Beckman and Beckman (1967), while the 100,000–125,000 fraction contained B, D and

some A band material. The prostatic enzyme electrophoreses as an "A" band. This would mean that the seminal ACP would be composed of two α polypeptide chains, as defined by Swallow and Harris (1972), and would be coded for by the ACP_3 locus (Swallow *et al.*, 1973).

Seminal plasma and prostate contain only one of the MW classes of ACP, the MW of the enzyme being about 100,000 (see in section 10.3.6). The enzyme exhibits molecular heterogeneity upon being subjected to starch gel electrophoresis (Sur *et al.*, 1962), but this multiplicity of forms derives from the attachment of varying numbers of sialic acid residues to the same protein. The molecular heterogeneity is thus post-synthetic, and not genetic, in origin (Smith and Whitby, 1968; and see section 10.3.6).

Lysosomes contain acid phosphatase enzymes of the 100,000–130,000 MW class. The rat liver enzyme was purified and studied by Brightwell and Tappel (1968). There is no evidence for polymorphism in human lysosomal ACP, but a rare, autosomally inherited deficiency of the enzyme has led to significant pathological findings, and early death in one family (Nadler and Egan, 1970).

29.3.3 Linkage relations of the *ACP* loci

ACP_1, which determines the polymorphic red cell isozymes, is not closely linked to any of the principal blood group or serum protein marker system loci (Conneally *et al.*, 1965). ACP_1 was found to be syntenic to soluble isocitrate dehydrogenase by somatic cell hybrid studies (Povey *et al.*, 1974), thus assigning it to chromosome 2. A peculiar deletion in a family allowed further localization of the ACP_1 locus to the short arm of chromosome 2. The ACP_2 locus was found to be syntenic with LDH-A, and thus assigned to chromosome 11, by human-rodent somatic cell hybrid studies (Bruns and Gerald, 1974).

29.4 Studies on the ACP_1 Isoenzymes (EAP Isoenzymes)

The earlier studies on the red cell ACP (Abul-Fadl and King, 1948 and 1949; Tsuboi and Hudson, 1953, 1954 and 1956) were carried out on mixtures of isoenzymes since the polymorphism had not yet been recognized. Some of the findings concerning inhibitors and substrate specificities of the red cell enzymes are quite useful in distinguishing them from other kinds of acid phosphatases.

Hopkinson *et al.* (1964) and Spencer *et al.* (1964a) determined the mean enzymatic activity of a number of individuals of the five common phenotypes, and found significant differences. They used p-nitrophenyl phosphate as the substrate. The results indicated that the ACP_1^A allele gave a product with about 61 units of activity, ACP_1^B and ACP_1^C yielding products with 94 and 120 units, respectively, where a unit was defined as 1 μmol p-nitrophenol liberated/30 min/g hemoglobin at 37°. Scott (1966) studied the kinetic properties of the enzymes from A and B homozygotes, and found only slight differences. His finding that ACP-A cells exhibit about 65% of the activity of ACP-B cells is fully confirmatory of the findings of Hopkinson *et al.* (1964). The differences in kinetic constants were not alone sufficient to explain the activity differences. Luffman and Harris (1967) carried out further extensive biochemical studies on the red cell isozymes. Although each isoenzyme differed somewhat in its substrate specificity, there was a clear overall pattern showing that all the isozymes were most active with p-nitrophenyl phosphate and FMN. Phenyl phosphate was a poorer substrate, and 3-phosphoglycerate, α- and β-glycerophosphates, 6-phosphogluconate, glucose-6-phosphate and methyl phosphate were all less active. The α-glycerophosphate was a better substrate than the β-isomer. Glc-1-phosphate, 2,3-diphosphoglycerate, ATP and AMP were all inactive. Tsuboi and Hudson (1956) had obtained similar results concerning substrate specificity with a better purified preparation, except that p-nitrophenyl phosphate was not tested, and peculiarly, FMN was found to be only about 40% as active as phenyl phosphate. Luffman and Harris (1967) also confirmed the earlier findings of Tsuboi and Hudson (1953) that the red cell isozymes have considerable phosphotransferase activity, and that a number of alcohols can act as phosphate acceptors in place of water. Glycerol was the best of the alcohols, followed by methanol, propanol and ethanol. Sensabaugh and Golden (1976a) carried out studies on the phosphotransferase activity of ACP, and extended the range of alcohols that had been tested as acceptors. They showed that the overall rate of the reaction was limited by the transfer of the phosphate group from the phospho-enzyme to water, and that the transfer to alcohol proceeded along a parallel pathway, and was additive in terms of the rate of the over-all reaction. Glycerol, and molecules containing a glycerol moiety, were found to be the most effective acceptors of all the alcohols tested. These findings have been exploited in devising better detection procedures for ACP on gels following electrophoresis. This matter is discussed in section 29.5.2. Thermal denaturation studies by Luffman and Harris (1967) indicated that the ACP_1^A products were most stable to heating, followed by those of ACP_1^B and ACP_1^C, in that order. Urea or guanidine caused denaturation of all the isozymes equally effectively. Rogers *et al.* (1978) showed that activity of ACP A and ACP B in red cells decreased at different rates as the cells age *in vivo*, but no secondary isozyme formation was noted during the aging process.

Fisher and Harris (1969) reported on the purification and characterization of the red cell isoenzymes in some detail. An effort was made to devise a purification procedure which was adaptable to large scale isolations. The results of this effort, which had been successful, were given by Fisher *et al.* (1968), with special reference to the ACP-B isozymes. Fisher and Harris (1969) found that inorganic phosphate stabilized the enzyme, and the addition of inorganic phosphate at various stages of the purification procedure improved the yield considerably. In 1964, Bottini and Modiano reported that the electrophoretic mobility of red cell ACP isozymes was strongly affected by sulfhydryl reagents. The addition of oxidized glutathione caused significant increases in the anodal mobility of the isozymes. Fisher and

Harris (1969) further investigated this effect, and found that oxidized glutathione did indeed lead to significant increases in mobility for all the types. A similar but less drastic effect was seen with lysates that had been aged. The presence of mercaptoethanol caused the isoenzymes to retain the mobility seen in fresh samples, and the reagent was thus routinely added to preparations of the isozymes. The enzymes apparently undergo oxidation of the -SH groups upon aging, and the effect can be mimicked in part by the addition of oxidized glutathione, though the glutathione effect is more drastic.

Dissing *et al.* (1979) tested a series of phosphonic and arsonic acids as inhibitors for ACP isozymes. Those which bound the enzymes well could then be used as ligands to design affinity chromatographic purification steps for the isozymes. There is some information on the properties of the less common red cell ACP enzymes. Jenkins and Corfield (1972) found that the products of ACP_1^R have about the same activity as those of ACP_1^A, and that the ACP_1^R isozyme was somewhat more thermostable than the ACP-A or ACP-B enzymes. Sørenson (1975) found that the products of the ACP_1^E locus were somewhat more active than those of other known alleles, and the ACP-E isozyme was more heat stable than the ACP-B one.

There have been some studies comparing the different products of the same ACP_1 allele. The apparent production of more than one product by a single allele, while far from unprecedented, wants some explanation. Hopkinson and Harris (1967) found that two principal isozymes could be separated by DEAE-cellulose column chromatography from each of the homozygous phenotypes AA and BB. Fenton and Richardson (1967) said that three distinct activities could be separated from red cells of any of the phenotypes by DEAE-Sephadex chromatography. These differed in a number of their properties, as well as in electrophoretic mobility. The five common ACP phenotypes contained different percentages of the three activities. In 1971, White and Butterworth purified the isoenzymes from red cells of the B and BA phenotypes, and found that they could be resolved into 5 and 7 components, respectively, which had similar substrate specificities, but differed in K_m and pH optima (White and Butterworth, 1971a and 1971b). The MW for the B components was estimated to be 13,000 by gel filtration, a value in reasonable agreement with the estimate of Luffman and Harris (1967) of 7,000 to 10,000 by the same technique but on cruder material. Fisher and Harris (1971) purified the separate isozymes from particular loci, and carried out studies on their characteristics. Isozyme products of the ACP_1^A locus are designated a_1 (slower) and a_2 (faster), and likewise, those of ACP_1^B are b_1 and b_2. The a_2 isozyme had a lower K_m than a_1 for p-nitrophenyl phosphate, and b_2 had a lower K_m than b_1 with the same substrate. If pure preparations of b_1 or b_2 were kept at 4°, and examined subsequently by electrophoresis, a "storage band" had developed that was identical to the other isozyme. Put another way, pure preparations of either b_1 or b_2, stored for a time, gave a $b_1 + b_2$ pattern upon electrophoresis. This transition occurred more rapidly in the absence of inorganic phosphate. There were indications that a_1 and a_2 might be interconvertible as well. This evidence suggests that the a_1-a_2 and b_1-b_2 pairs might be conformational isomers of one another. If so, the apparent production of more than one isozyme by a single genetic locus can be readily understood.

Kaczmarek (1976) reported that red cell ACP isozymes could be resolved into some 21 zones of activity by isoelectric focusing, and further, that neuraminidase treatment abolished the multiple banding and reduced the activity to a single band of ACP activity with a pI of 5.8. This observation is not in accord with the data of White and Butterworth (1971a) nor with that of McWright *et al.* (1975). The molecular weight data on the isozymes are not readily understandable in terms of the values determined in a number of other laboratories either.

The properties of acid phosphatases, including those of the red cell and of tissues, were reviewed by Hollander (1971).

29.5 Medicolegal Applications

29.5.1 Disputed parentage

The ACP system has been employed in disputed parentage cases in a number of laboratories since about 1966. A number of investigators have established that the ACP_1 system is fully reliable in these cases (Fuhrmann and Lichte, 1966; Fiedler, 1967; Speiser and Pausch, 1967; Broman *et al.*, 1971; and others). The isoenzymes are fully expressed in the newborn (Reimann and Römisch, 1968) as well as in fetal blood (Chen *et al.*, 1977). Jarosch (1968) discussed the use of ACP in paternity cases. Speiser and Pausch (1967), Krüger *et al.* (1968) and Hummel *et al.* (1969) gave accounts of the calculations of the probabilty of excluding a falsely accused man.

The probability of excluding a falsely accused western European man with the ACP system alone is about 25%. Speiser and Pausch (1967) quoted a value of 24.98% for the Viennese population, Broman *et al.* (1971) said that the figure was 25.9% for Sweden, and Boorman *et al.* (1977) gave 21% for the British population. Polesky *et al.* (1976), gave figures of 25.08% for Caucasians, and 15.44% for Blacks, and these were calculated from U.S. population data.

The possibility of encountering a silent allele in a parentage case should be kept in mind. Heide *et al.* (1974) discussed this problem, and said that quantitative enzyme assays could be employed in cases of apparently contradictory homozygosity. The silent allele of ACP_1 is extremely rare (section 29.2).

It should be kept in mind, too, that problems with ACP_1 typing can occasionally be encountered in "fresh" blood samples, as in the case reported by Andrus (1980) and discussed in section 29.5.2.

29.5.2 ACP phenotyping in dried bloodstains

Heidel (1968) reported that ACP_1 phenotypes were reliably determinable in dried bloodstains up to 30 days old.

Smerling (1969) agreed that the system could be typed in dried bloodstains, but only if they were quite fresh. No results could be obtained with stains more than about 30 hours old. Nagata and Dotzauer (1970) could not obtain results with bloodstains older than about 30 hours either. They investigated dried stains on a number of different substrata. If the stains were kept at −40°, the enzyme was preserved better, and could be typed in stains up to 80 hours old. Brinkmann (1971) found that stains kept at room temperature could be typed for up to 3 weeks by polyacrylamide gel electrophoresis. Subsequent investigations showed that such stains could be phenotyped for up to 6 or 8 weeks (Brinkmann *et al.* (1972b). The polyacylamide gel method used in these studies was described by Hennig *et al.* (1968). There were differences in the time limits of detection of the isoenzymes in bloodstains in different phenotypes, and at different storage temperatures. There were differences, too, between stains absorbed into substrata and dried blood on polished surfaces, which could be scraped off. All types could be determined at 60 days with blood crusts, after which A, CA and BA became difficult to distinguish because of the lability of a_2. Types B, CB and C could be determined after 12 weeks. In absorbed stains, all the phenotypes could be determined at 6 weeks, and the B, CB and C types at 8–9 weeks. Stains of all kinds stored in the deep freeze were stable for considerably longer periods of time (7½ months). It should be noted that absorbed stains were extracted in 60 mM phosphate buffer, pH 6.2, containing 60 mM mercaptoethanol. The extracts were then lyophilized, and the lyophilizate redissolved in minimal 30 mM phosphate buffer, pH 6.2, containing 30 mM mercaptoethanol. Blood crusts were dissolved in the 60 mM phosphate buffer, pH 6.2, 60 mM mercaptoethanol solution for three hours time.

A major study on bloodstain grouping of ACP was carried out by McWright *et al.* (1975). Starch gel electrophoresis was employed using the citrate-phosphate buffer system of Hopkinson and Harris (1969a) modified to include 20 mM $MgCl_2$ and 10 mM EDTA, and prepared at pH 5.5. The detection substrate was 4-methylumbelliferyl phosphate. Consistently better patterns were obtained after 4 hours of electrophoresis than after 15 hours. "Storage" bands were observed with all the types. One of these, associated with B, BA and CB was always present; another, associated with A, was present in A types but virtually absent in BA and CA types. The intensity of such storage bands is lessened by treatment of the samples with sulfhydryl reagents (0.1M dithiothreitol was used in this case). All phenotypes could be determined in bloodstains for up to 4 months, after which certain phenotypes became difficult. Somewhat in contrast to the findings of Brinkmann *et al.* (1972b), the CB and CA were found to be more stable than the A, B and BA types. Occasionally, it was noted that the faster isozymes of B and CB types could become weaker upon aging, while the slower isozymes become slightly more intense. In at least one instance, a B would have been read as a weak C in a four day old stain. Densitometric tracings of the phenotypic patterns were employed to supplement visual judgments throughout the study. Neuraminidase treatment of lysates for lengthy periods destroyed ACP activity, but did not lead to any alteration in the phenotypic patterns.

In 1976, Wraxall and Emes reported a starch gel electrophoretic method for ACP typing in bloodstains using the citrate-phosphate buffer system of Hopkinson and Harris (1969). Electrophoresis was carried out for 4½ hours on cooling platens, and methylumbelliferyl phosphate was used as a detecting substrate. Samples were treated with 50 mM dithiothreitol prior to electrophoresis. A storage band was noted in hemolysates, but this was removed in most cases by the DTT treatment. The storage band was not normally seen in dried bloodstains. Blind trial studies indicated that the procedure was completely reliable. Some older stains (up to 6 weeks), particularly those on synthetic fabrics, gave weak or negative results. Heated lysates can yield incorrect results, because the a isozymes are least stable, followed by the b, and then the c ones. A type B pattern can thus be induced to take on the appearance of a CB, and ultimately of a C, but these changes were not observed in aging bloodstains. Similar kinds of effects were observed by Berg *et al.* (1974b) in their studies on the effect of heating on ACP phenotype determination. Wraxall and Emes (1976) emphasized that care must be taken to insure that excess heating does not occur during the electrohoretic run. Caution in the interpretation of bloodstain patterns was recommended as well, especially in cases of older stains. It appears that Figures 2 and 5 in this paper were inadvertently interchanged in the printing process.

Sensabaugh and Golden (1976a) suggested a modification of in the detection reaction procedure for ACP, based upon their studies of the phosphotransferase activity of the enzyme. The phosphotransferase activity of ACP was noted by Tsuboi and Hudson (1953), and confirmed by Luffman and Harris (1967), the latter of whom studied this activity with a number of alcohols. Sensabaugh and Golden (1976a) extended the studies to include a wider range of alcohols, and could show that glycerol, and structures containing a glycerol moiety, gave the best rates. Taking advantage of this effect for the detection of ACP phenotypes following electrophoresis, they found that the incorporation of glycerol into the starch gel enhanced the rate of isozyme development considerably, with methylumbelliferyl phosphate as substrate. The bands were more compact, and mobility was decreased presumably because of the increased viscosity of the gel in the presence of glycerol. The optimal concentration of glycerol was 15% (v/v) in gel buffer. This interesting approach is, however, not without its problems and pitfalls. Sensabaugh and Wraxall (1977) took up the subject again, and pointed out several other problems associated with reliable ACP phenotyping. Since the activity of the various isozymes is quite different (section 29.4), i.e., the different isozymes differ in their K_m for substrate, the glycerol enhancement effect is differential. Because the differentiation of some ACP phenotypes on electrophoretic

plates is a matter of band intensity differences, misinterpretation of phenotypes is possible if these effects are not fully appreciated. It is essential, therefore, to include appropriate controls of known phenotypes when using any phenotyping procedure, and particularly when attempting to diagnose B, CB and C phenotypes. They also noted the importance of using substrate concentrations high enough to insure that all the isozymes would be saturated, since the K_m values are not the same for all the isozymes, and misleading intensity differences could be produced by using substrate concentrations that are too low.

In 1978, Grunbaum and Zajac reported that ACP phenotypes could be determined in bloodstains up to 30 days old, as well as on hemolysates, by electrophoresis on cellulose acetate membranes. These investigators reported a blind trial study on ACP phenotyping on starch gels and on cellulose acetate foils (Zajac and Grunbaum, 1978), in which bloodstains, hemolysates, and hemolysates which had been kept at 37° for up to 48 hours, were all tested. Samples of BA, B and CB phenotypes were determined on both kinds of media, and those of A and CA types were determined on cellulose acetate membranes. Stain phenotypes were correctly determined, with the exception of one "inconclusive" result on a BA stain on cellulose acetate, and of one CB stain. The CB could be confused with type B on either support medium. The heated samples caused problems in interpretation on starch or cellulose acetate, except for CB phenotypes. While the heated samples were correctly typed in some instances, they were misinterpreted in others. B was mistyped as CB, BA as CA or CB, and some results on heated lysates were inconclusive. Caution was recommended in cases where the history of the sample was not very well known.

Welch (1972b) found that ACP could be determined in 22 days old bloodstains, but not in 30 days old ones. Denault *et al.* (1978) found ACP phenotypes to be detectable in bloodstains on a variety of substrata for up to 13 weeks. In one case, a stain on cotton did not give a result after 4 weeks aging. Stains aged at higher humidities were generally more active than those kept at lower humidity. One B stain on denim, a BA stain on a perma-press fabric, and another stain on cotton kept frozen could all be typed after 26 weeks. Only B and BA stains were included in the study.

Brinkmann and Bruns (1979) conducted phenotyping tests on two series of bloodstains, representing all the phenotypes, one on cotton and the other on glass. Members of each series were kept at 37°, 22°, 4° and frozen. Electrophoresis was carried out on thin layer agarose gels as well as on Cellogel. The stains on glass were typable longer than those on cotton. The stains at room temperature were typable for 3 to 6 weeks. Those at 37° lasted only a few days. In the refrigerator and freezer, stains could be typed for 10 to 13 weeks. The Cellogel was preferred over the agarose gel, and was said to give clear, reliable typing results.

Andrus (1980) reported an interesting case in which a typing problem was encountered not with the dried blood (which was on window glass), but with the "fresh" blood sample taken from the suspect. The blood had been drawn into EDTA 4 days prior to its receipt by the lab, and had been kept in a refrigerator. There was little observable hemolysis, and the red cells from the sample and the bloodstains were typed for ABO, PGM and PGM subtypes, ESD, AK, ADA, GLO, Hp and Gc. The bloodstain yielded a type B for ACP_1, however, while the cells yielded CB. The subject had not been transfused at all recently, if ever. A second sample of blood from the same person, collected in EDTA and examined within 24 hours, revealed ACP type B. The other systems typed as they had in the original sample. There was no indication that the original blood sample had been mishandled, heated or improperly stored.

29.5.3 Methods of phenotyping ACP isoenzymes

Most investigators have used starch gel electrophoresis for typing acid phosphatase isoenzymes, although a variety of different buffer systems have been described. The buffer system employed in the original paper on the ACP_1 polymorphism was described in section 29.2. A discussion of buffer effects on phenotypic patterns was given by Hopkinson and Harris (1969), and was reviewed in section 29.2 as well. Radam and Strauch (1966) proposed a discontinuous buffer system for starch gel electrophoresis consisting of 12 mM phosphate, pH 6, gel buffer, and 0.4M citric acid-NaOH, pH 6, bridge buffer. Methods have been devised for phenotyping ACP on agarose (Sørensen, 1974a), on polyacrylamide gels (Hennig *et al.*, 1968), and on cellulose acetate foils (Grunbaum and Zajac, 1978) and Cellogel (Brinkmann and Bruns, 1979). Brinkmann *et al.* (1972b) used the horizontal polyacrylamide gel system of Hennig *et al.* (1968). It should be noted that the gels were prepared with DMAPN, potassium ferricyanide and ammonium persulfate, and not with TEMED, and photopolymerized. They were then soaked in gel buffer for a number of days, with several changes of buffer, in order to rid the gel of any small molecule inhibitors. The process was called "diffusion washing". This matter was discussed in section 27.4.2.1 as well. In the case of ACP, 3% gels were used. Less material was required with the system than with starch. Brinkmann and Bruns (1979) have more recently tested Cellogel and thin-layer agarose gels, and said they preferred the Cellogel. Isoelectric focusing has been used for ACP typing. Sørensen (1974b) used the technique to measure the isoelectric points of the isoenzyme components. Burdett and Whitehead (1977) reported good phenotyping results by isoelectrofocusing in polyacrylamide gels in pH 5.0–8.5 gradients.

A number of systems have been devised in which ACP may be phenotyped simultaneously along with other isoenzyme systems. Hummel (1970) described a system for ACP with PGM. Martin and Niebuhr (1971) gave a procedure for typing ACP along with PGM, AK and ADA in the same electrophoretic operation. Wraxall and Stolorow (1978) have recently described a system designed for dried bloodstains in which ACP can be phenotyped along with AK and ADA in the same gel. They refer to these three iso-

zyme systems as "Group II", to distinguish them from other multi-system procedures they have devised. Their "Group I" consists of ESD, PGM and GLO, and is mentioned in connection with the discussions of those systems.

Acid phosphatase assays were discussed in section 10.3.4, and most of the substrates used for phosphatases were covered in that section. The red cell enzyme shows a narrower range of substrate specificity than is characteristic of acid phosphatases in general. Most investigators used phenolphthalein diphosphate as the substrate for detecting ACP in gels up until fairly recently. For enzymatic assay of the ACP, p-nitrophenyl phosphate is often preferred because the p-nitrophenylate anion is so easy to detect spectrophotometrically. Sørensen's (1970) finding that β-naphthyl phosphate was a suitable substrate, provided that the coupling dye was not added simultaneously (section 29.3.2), caused some workers to switch to this system. The availability of the fluorigenic substrate 4-methylumbelliferyl phosphate has resulted in its being adopted as the ACP substrate in a number of the more recent studies. Sparkes *et al.* (1975) said that phenolphthalein monophosphate was as sensitive and as satisfactory as the MUP for red cell ACP typing, and had the advantage that exposure to UV light was not required to visualize the zones of activity.

29.5.4 Red cell ACP typing in aged whole blood samples

There are conflicting reports in the literature on the survival of typable ACP isozymes in aging blood samples, and in postmortem specimens. Like the comparable reports on the survival of the enzyme in bloodstains, some of these differences can probably be accounted for by variations in electrophoretic technique and/or in the substrate used to detect the isoenzymes. Reimann and Willner (1968) said that ACP was typable for a number of months in samples collected for blood alcohol determinations. Smerling (1969) found the time limit to be at least 6 months. Gussmann (1970) found that, after a month or two, the C phenotypes were very difficult to determine accurately. Brinkmann *et al.* (1972b) found that samples kept at 4° could be determined for up to 15 months, but increasingly larger quantities of sample had to be used as the blood aged. Rose (1971) noted problems with CB type blood after only 11 days storage, and said that the formic acid system of Giblett and Scott (1965) gave better differentiation of CB and CA in these samples. McWright *et al.* (1975) found that hemolysates or clotted blood kept at 25° lost activity after about 5 days. Blood in citrate-phosphate-dextrose anticoagulant at 5°, however, could be typed after 10 months.

Herzog and Sobotka (1972) said that ACP had been typed in postmortem specimens up to 5 days after death. Heidel and Reimann (1968) could type such samples up to a month old on some occasions.

Krauland and Smerling (1971) reported a most peculiar differential inhibition of red cell ACP by a commercial chemical contained in blood-drawing containers. The chemical was called "polyanetholsulfonsaurem natrium", and was present at a concentration of 1% in physiological saline. The compound is probably a poly-anethol sulfonic acid (sodium salt) of some sort (anethol is p-methoxypropenylbenzene). In any event, the A isozyme was differentially inhibited, so that BA could be taken for B in the presence of this material.

29.6 Distribution of ACP_1 phenotypes in U.S. populations

The data are presented in Table 29.1. ACP is one of the more useful systems for making distinctions in the population. The discrimination index is about 0.65 for Caucasians, and about 0.54 for Negroes.

Table 29.1 Distribution of *ACP*$_1$ Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) A	BA	B	CA	CB	C	Rarer	ACP_1^A	ACP_1^B	ACP_1^C	Reference
CAUCASIAN												
Seattle, WA	193	33 (17.1)	76 (39.4)	61 (31.6)	10 (5.2)	13 (6.7)	0		0.394	0.547	0.059	Giblett and Scott, 1965
U.S. Navy Personnel in Japan	272	27 (9.9)	120 (44.1)	107 (39.3)	7 (2.6)	11 (4.0)	0		0.3327	0.6342	0.0331	Omoto and Harada, 1968
Chicago, IL	100	14 (14)	43 (43)	38 (38)	4 (4)	1 (1)	0		0.375	0.600	0.025	Shih and Hsia, 1969
Pittsburgh/Allegheny County, PA	1,239	145 (11.7)	526 (42.5)	491 (39.6)	20 (1.6)	57 (4.6)	0		0.337	0.632	0.031	Hagins et al., 1978
California	4,850	(10.8)	(42.1)	(39.3)	(3.3)	(4.3)	(0.2)		0.332	0.630	0.038	Grunbaum et al., 1978b
Bexar County, TX	200	(12.0)	(36.0)	(50.0)	(>1)	(1.0)	(0)		0.310	0.690	0.010	Ganaway and Lux, 1978
Detroit, MI	503	65 (12.9)	185 (36.8)	193 (38.4)	22 (4.4)	38 (7.6)	0		0.335	0.605	0.060	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade Co., FL	366	44 (12.0)	145 (39.6)	156 (42.6)	10 (2.7)	10 (2.7)	1 (0.3)		0.330	*0.640*	*0.030*	*Stuver, 1979* and see Shaler, (1978)
Los Angeles, CA Case material	357	34 (9.5)	150 (42.0)	158 (44.3)	8 (2.2)	7 (2.0)	0		0.317	0.662	0.021	Sigler, 1979 and see Shaler, (1978)
NEGRO												
Seattle, WA	164	12 (7.3)	48 (29.3)	99 (60.4)	2 (1.2)	3 (1.8)	0		0.226	0.759	0.015	Giblett and Scott, 1965
Ann Arbor, MI	224	12 (5.4)	50 (22.3)	160 (71.4)	0	2 (0.9)	0		0.1651	0.8303	0.0044	Brewer et al., 1967a
Austin, TX	63	3 (4.8)	16 (25.4)	32 (50.8)	1 (1.6)	8 (12.7)	0	★	0.19	0.71	0.07	Karp and Sutton, 1967
Dallas/Houston, TX Male patients	294	11 (3.7)	100 (34.0)	166 (56.5)	1 (0.3)	7 (2.4)	0	☆	0.21	0.76	0.015	Karp and Sutton, 1967
Seattle, WA	425 ✪	30 (7.0)	150 (35.0)	222 (51.7)	2 (0.5)	10 (2.3)	0	●	0.25	0.72	0.014	Giblett, 1969 and Mourant et al., 1976
Chicago, IL	101	8 (7.9)	19 (18.8)	66 (65.3)	1 (1.0)	2 (2.0)	0	◇	0.181	0.761	0.015	Shih and Hsia, 1969 and Mourant et al., 1976
Pittsburgh/Allegheny County, PA	718	39 (5.4)	239 (33.3)	426 (59.3)	2 (0.3)	11 (1.5)	0	□	0.222	0.767	0.009	Hagins et al., 1978
California	875	(5.6)	(31.4)	(60.2)	(0.2)	(1.3)	(0.1)	(1.1)	0.217	0.776	0.008	Grunbaum et al., 1978b

Table 29.1 (Cont'd)

Population	Total	Frequency — Number (Percent) A	BA	B	CA	CB	C	Rarer	ACP_t^A	ACP_t^B	ACP_t^C	Reference
Bexar County, TX	200	(7.0)	(27.0)	(66.0)	(0)	(0)	(0)		0.205	0.795	–	Ganaway and Lux, 1978
Detroit, MI	504	30 (6.0)	171 (33.9)	280 (56.0)	4 (0.8)	6 (1.2)	0	○	0.234	0.742	0.011	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade Co., FL	345	24 (7.0)	110 (31.9)	201 (58.3)	1 (0.3)	6 (1.7)	0	/	0.229	0.743	0.010	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	161	5 (3.1)	66 (41.0)	89 (55.3)	0	1 (0.6)	0		0.236	0.761	0.003	Sigler, 1979 and see Shaler, (1978)
MIXED NEGRO AND CAUCASIAN												
Washington, D.C.	137	14 (10.2)	52 (38.0)	61 (44.5)	4 (2.9)	6 (4.4)	0					McWright et al., 1975
ORIENTAL												
Seattle, WA ("mixed")	77	4 (5.2)	22 (28.6)	51 (66.2)	0	0	0		0.195	0.805		Giblett and Scott, 1965
	221 ○	8 (3.6)	70 (31.7)	142 (64.3)	0	1 (0.5)	0		0.2	0.8	0.002	Giblett, 1969 and Mourant et al., 1976
ASIAN												
California and Hawaii	2,462	(5.2)	(35.6)	(59.2)	(0)	(0)	(0)		0.23	0.77	–	Grunbaum et al., 1978b
HISPANIC												
California ("Chicano/ Amerindian")	1,360	(6.7)	(35.8)	(53.5)	(1.8)	(2.2)	(0)	(0.2)	0.264	0.726	0.019	Grunbaum et al., 1978b
Bexar County, TX	200	(9.0)	(31.0)	(60.0)	(2.0)	(0)	(0)		0.235	0.765	0.010	Ganaway and Lux, 1978
Miami/Dade Co., FL	362	27 (7.5)	123 (34.0)	184 (50.8)	7 (1.9)	20 (5.5)	1 (0.3)		0.254	0.706	0.040	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	179 ■	6 (3.4)	69 (38.6)	99 (55.3)	2 (1.1)	3 (1.7)	0		0.232	0.764	0.014	Sigler, 1979 and see Shaler, (1978)

★ 1 RA and 2 RB ☆ 1 RA, 5 RB and 1 RC ○ May include the data of Giblett and Scott, 1965 ● 3 RA and 11 RB ◇ 5 RB □ 1 RA ○ 1 RA, 11 RB and 1 RC / 1 DB, 1 RB, and 1 RA ■ Primarily Mexican

SECTION 30. ADENOSINE DEAMINASE

30.1 Recognition of Adenosine Deaminase

Adenosine deaminase (Adenosine aminohydrolase; E.C. 3.5.4.4; ADA) is an enzyme of nucleoside catabolism, and catalyzes the conversion of adenosine to inosine with the liberation of a mole of ammonia. The enzyme occurs in the tissues of a wide variety of invertebrates and vertebrates.

Schmidt (1928 and 1932) provided the first systematic evidence for purine deaminase activities in rabbit skeletal muscle and liver. These studies included adenylic acid deaminase and adenosine deaminase. Conway and Cooke (1939) found adenosine and adenylic acid deaminase activities in the blood and tissues of rabbits. Brady (1942) found that the superficial mucosa of calf intestine was a rich source of ADA, and this enzyme was found to deaminate deoxyadenosine about as well as it did adenosine. The human blood enzyme was said to be only about half as active with deoxyadenosine as with adenosine. The calf intestinal mucosa enzyme was extensively purified by Brady and O'Connell (1962). The enzyme behaved homogeneously in the ultracentrifuge, but showed molecular heterogeneity upon starch gel electrophoresis. This enzyme has been studied quite extensively (Zielke and Suelter, 1971 for a review).

30.2 ADA Polymorphism in Human Red Cells

In 1968, red cell ADA was found to exhibit regular starch gel electrophoretic patterns which were different in different individuals (Spencer *et al.*, 1968). Three phenotypes were recognizable, and family studies indicated that these could be accounted for by a pair of codominant alleles, ADA^1 and ADA^2, at an autosomal locus. The ADA phenotypes were called 1, 2-1 and 2. The ADA^2 frequency was low in European Caucasians and in Negroes, but somewhat higher in Asiatic Indians. In 1969, Hopkinson *et al.* carried out further population and family studies, which were fully consistent with the single allelic pair hypothesis. This genetic model has been confirmed by a number of other population and family studies (e.g. Tariverdian and Ritter, 1969; Renninger and Bimboese, 1970).

A new phenotype was seen by Hopkinson *et al.* (1969) which was similar to an ADA 2-1, but with considerably reduced activity. The phenotype was attributed to heterozygosity between ADA^1 and a new, rare allele, called ADA^3. The rare allele, ADA^4, was detected as an ADA 4-1 by Dissing and Knudsen in 1969. The main isozyme conditioned by ADA^4 is slower (less anodal) than those of the other three alleles. Detter *et al.* (1970b) found a "fast" ADA pattern in two unrelated Black Americans in Seattle, and provisionally named the phenotype ADA 5-1. The subjects were unavailable for further study to prove the segregation of the postulated ADA^5 allele. Renninger and Bimboese (1970) found an unusual ADA phenotype in an African Black individual who was a member of the tribe of Macua of Moçambique. They did not give the new phenotype a designation, but it looks very much like the ADA 5-1 described by Detter *et al.* (1970b) in Seattle, although the specimens were never directly compared. Radam *et al.* (1974) described a family with yet another phenotype, called ADA 6-1, and family studies showed that the rare allele ADA^6 was segregating in a number of members. A diagrammatic representation of the ADA phenotypes, as seen by starch gel electrophoresis, is given in Figure 30.1. The single allelic pair hypothesis of inheritance for the common ADA phenotypes has been widely confirmed (e.g. Dissing and Knudsen, 1970; Lamm, 1971a).

Silent alleles have been reported at the *ADA* locus. The situation is somewhat complicated because it appears that not every example of deficiency of ADA activity in red cells has the same genetic basis. In 1972, Giblett *et al.* made the extraordinary observation that two unrelated young girls, both suffering from immunodeficiency disease, had no detectable ADA in their red cells. The probability that two such extremely rare conditions could occur in two unrelated individuals by chance alone was too great to be regarded as reasonable. The parents of one of the girls had about half as much ADA activity as normal cells, and the parents of the other girl about two-thirds as much. This report was quickly followed by another from Dissing and Knudsen (1972) that a six month old girl had been observed with combined immunodeficiency disease (CID) and an absence of red cell ADA activity. She was very ill, and did not survive. A young boy with CID, however, showed normal red cell ADA activity, as did his parents. These investigators thought it might be significant that the three CID-ADA-deficient subjects seen thus far had been female. But in 1974, Chen *et al.* found a little boy with CID and ADA deficiency, and studies on the family indicated that an apparently silent allele for ADA was segregating in four generations. The pedigree of this interesting family is shown in Figure 30.2. In 1973, Brinkmann *et al.* encountered an apparent mother-child ADA incompatibility in the course of a disputed paternity investigation. The mother was ADA 2, and three of her six children were ADA 1. Enzyme assays indicated that the mother and the three children had about 60% of the ADA activity of normal red cells, and the simplest explanation was heterozygosity for a silent allele, ADA^0. Everyone in this family was apparently healthy. Jenkins (1973) reported that he had found an ADA-deficient African boy in his studies of the blood groups of the !Kung, a group of bushmen in the northeastern part of southwest Africa. The boy

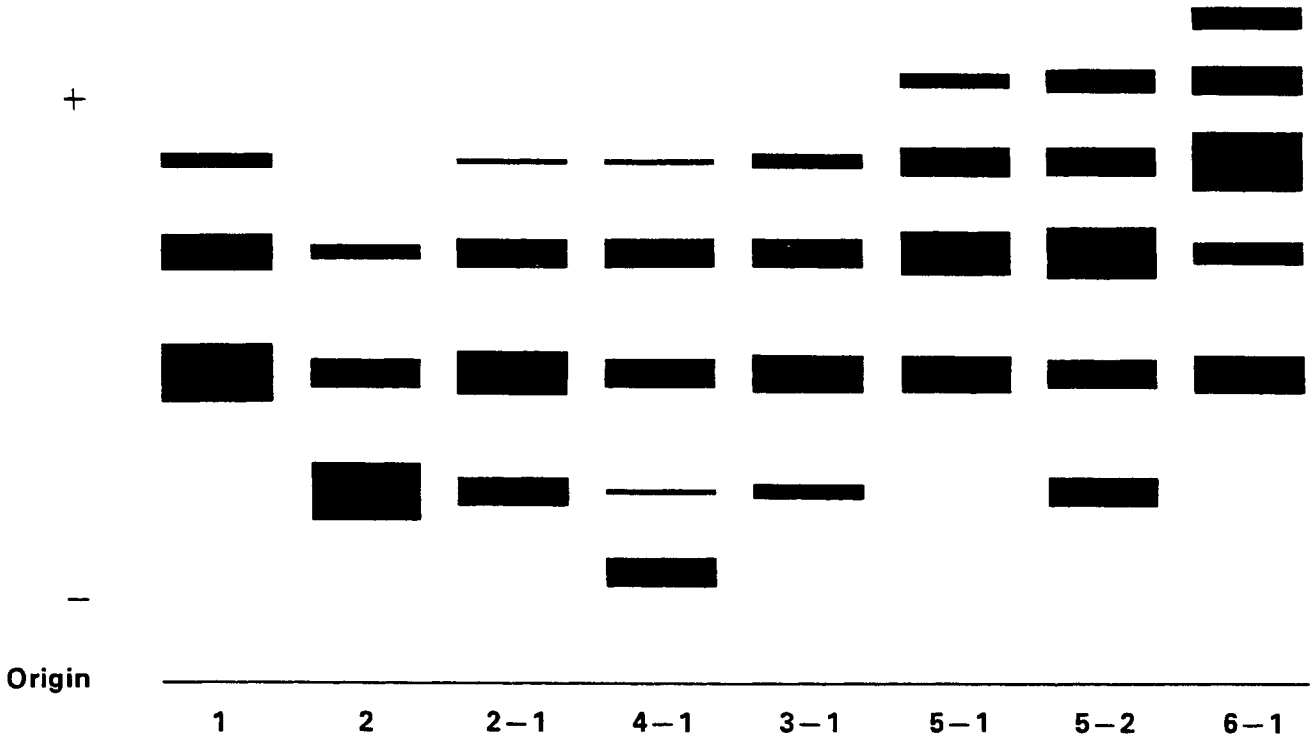


Figure 30.1 Electrophoretic Patterns of ADA Phenotypes

did not suffer from CID, and his father and sister showed reduced ADA activities. Additional studies on the !Kung have shown that an apparent silent allele for ADA reaches polymorphic frequency in these people (Jenkins *et al.*, 1979). In 1973, a conference was convened in Albany, NY, to bring together a group of interested experts for a discussion of the CID-ADA-deficiency relationship. A report may be found in Meuwissen *et al.* (1975). Fifty-five children with CID were known to the group, of whom 22 had had their red cell ADA tested. Thirteen of the 22 were ADA deficient. It appears, therefore, that there is more than one basis for ADA deficiency, as well as for CID. This subject is discussed further in section 30.3.

There is no pedigree evidence for close linkage of the *ADA* locus with the loci of other common blood groups, isozymes or serum proteins (Weitkamp *et al.*, 1970; Weitkamp, 1971). On the basis of human-rodent somatic cell hybrid studies, *ADA* has been assigned to chromosome 20 (Tischfield *et al.*, 1974).

30.3 ADA Isoenzymes in Other Tissues

Adenosine deaminase occurs in the tissues of a variety of mammals (Schmidt, 1928 and 1932; Conway and Cooke, 1939; Brady, 1942; Brady and O'Donovan, 1965). In 1969, Ressler found electrophoretically separable isozymes of ADA in a number of human tissues. Each tissue had a characteristic pattern, but changes in the patterns could be induced by heating the tissues *in vitro*. Ressler was inclined to the view that the isozymes might represent post-synthetically modified forms of a primary gene product. Akedo *et al.* (1970 and 1972) partially purified and characterized two molecular species of ADA from human tissues, distinguishable on the basis of molecular size, and thus separable by gel filtration. These forms were called "large" and "small" (MW about 230,000 and 47,000 respectively). Different tissues had different amounts of each, some tissues having primarily one or the other. There were no major differences in properties between the two, apart from heat stability. Denatured in guanidine, the large enzyme was converted into the small one; if this treated material was dialyzed, the large enzyme could be reconstituted. Therefore, either the large form was a polymer of the small form, or else it was constituted from the small form along with some other unidentified (and non-dialyzable) component.

In 1971, Edwards *et al.* investigated tissue adenosine deaminases. Most human tissues had ADA isoenzymes, and two sets of these could usually be distinguished. One of them was equivalent to "red cell" ADA, and showed the familiar polymorphism characteristic of erythrocyte ADA. The other isoenzymes, designated *a* through *e*, were heterogeneous, but showed a tissue-specific distribution pattern. The "red cell" isozymes in tissues resembled the isozymes from the actual red cells in MW as estimated by gel filtration (about 34,000 daltons) and in sulfhydryl reactivity

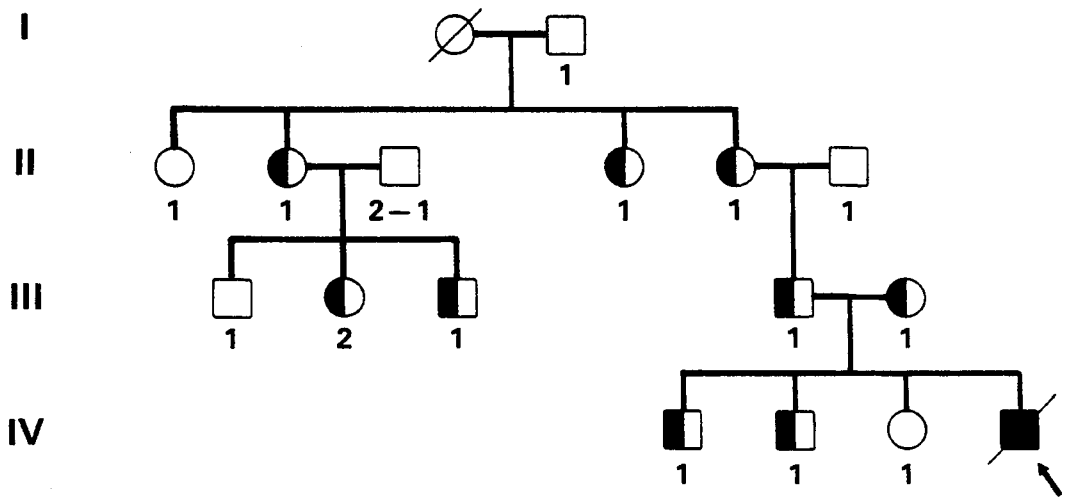


Figure 30.2 Segregation of Silent ADA Allele (after Chen et al., 1974)
ADA phenotypes indicated under each symbol: ◐ or ◧ are presumed heterozyotes for allele. ■ is homozygous for silent allele. Slash through symbol indicates deceased. Propositus indicated by arrow.

(see below). The tissue ADA isozymes differed from the "red cell" ones on both counts. The *b*, *c* and *d* isozymes were estimated to have MW of about 280,000, and the *e* isozyme, about 440,000;. the *a* isozyme was labile, and an estimate of its MW could not be obtained.

Van der Weyden and Kelley (1976) purified and examined ADA isoenzymes from human tissues. Four classes of enzyme could be distinguished on the basis of molecular size. One of these was "particulate" and had not been previously seen. The apparent MW was 20 million, and the activity was in some way associated with subcellular organelle membrane. This form was dissociable with nonionic detergent. The other three classes were soluble (i.e., not membrane-associated), and interconvertible, and had apparent MW's of 36,000 (small), 114,000 (intermediate) and 298,000 (large). The small form was converted to the large form in the presence of a protein with no ADA activity of its own and a MW of about 200,000. Conversion was best at 4°, and pH 5 to 8, and was associated with the loss of conversion activity. The small form of the enzyme predominated in tissues with little conversion activity, and conversely, the large form. Both small and large forms exhibited molecular heterogeneity upon isoelectric focusing. Apart from differences in pH optima, the small, intermediate and large forms were similar in their catalytic properties. Nishihara *et al.* (1973) isolated and purified the conversion factor about 600-fold, and characterized it further. It had a MW of about 139,000 as estimated by gel filtration, had no ADA activity of its own, and exhibited no sulfhydryl or metal requirements. The results of the study suggested that the large ADA molecule (MW 230,000) was some kind of a complex of the small ADA molecule (MW 47,000) and the conversion factor.

The interconvertibility of the "small" and "large" forms of the ADA isoenzymes, which was confirmed by Hirschhorn *et al.* (1974), suggests that they are all products of the same genetic locus. There is further evidence for this view as well. Hirschhorn *et al.* (1973) and Hirschhorn and Boratis (1973) examined the tissues, and cultured fibroblasts, of victims of the combined immunodeficiency disease-ADA deficiency syndrome which was discussed in section 30.2. Some of the tissues had been deep frozen at −70° for some time, but control tissues indicated that this treatment had no inhibitory effect upon ADA. No detectable ADA activity could be found in either the tissues or the cultured fibroblasts, suggesting that tissue as well red cell ADA isozymes were the products of the same genetic locus. In 1974, Hirschhorn *et al.* found that cultured fibroblasts from a patient suffering from CID-ADA deficiency syndrome had residual ADA activity. The residual enzyme was electrophoretically faster (more anodal) than control enzyme, but had a MW resembling that of the normal tissue enzyme (about 220,000). The enzyme from the patient differed somewhat in heat stability from the normal one as well, and these workers suggested that this residual enzyme was a "mutant" form of ADA. Chen *et al.* (1975) reported somewhat similar findings in their studies on the ADA from cultured fibroblasts of two unrelated patients with CID-ADA-deficiency syndrome. It is of some clinical interest that amniotic fluid cells in culture can be assayed for ADA

activity, and the "red cell" phenotype determined as well. This fact may be important in the application of amniocentesis to the prenatal diagnosis of CID-ADA-deficiency syndrome.

ADA activity in leucocytes was reported by Karker (1965), and was found to be some 30 times higher than in red cells on a "per cell" basis. The isoenzymes of white cells resemble those of heart, brain, muscle and spleen (Edwards *et al.*, 1971). Lymphocyte ADA isoenzymes were described by Wüst (1971a). The "red cell" isozymes are present in these cells, and the phenotype can be determined. There is an additional isozyme present, cathodal to the others. The pattern is similar to that reported by Edwards *et al.* (1971) for fibroblast ADA.

30.4 Purification and Properties of Red Cell ADA

30.4.1 Detection and assay of ADA

Assays for ADA in solution have been based upon following either the appearance of ammonia, or the disappearance of adenosine. The ammonia assay was used by the earlier workers (e.g. Brady, 1942). The disappearance of adenosine in nonturbid solutions can be followed at 265 nm (Kalckar, 1947), and ADA may be conveniently assayed in this way (Osborne and Spencer, 1973). More recent studies have employed radioactively-labelled adenosine for the ADA assay, since it is commercially available (van der Weyden and Kelley, 1976; Daddona and Kelley, 1977). ADA activity can be estimated in small amounts of dried blood by a micromethod that was recently described (Orfanos *et al.*, 1978).

The detection procedure for the ADA isozymes from crude tissue or hemolysate preparations involves a set of coupled enzymatic reactions. The original procedure, devised by Spencer *et al.* (1968), is widely employed. In it, the ADA reaction which brings about the conversion of adenosine to inosine is coupled through the nucleoside phosphorylase reaction to the xanthine oxidase reaction. MTT tetrazolium dye is reduced to formazan in the presence of PMS in the usual way as hypoxanthine is oxidized to xanthine. This detection reaction sequence is indicated in Figure 30.3.

30.4.2 Studies on red cell ADA

Hopkinson and Harris (1969b) noted that the isozyme pattern of ADA in hemolysates changed upon storage, and that these effects were accelerated by the addition of oxidized glutathione, but reversed by addition of mercaptoethanol or reduced glutathione. A detailed study of the sulfhydryl groups of ADA was carried out. Three different kinds of thiol reagents may be used to study reactive sulfhydryl groups in enzymes and proteins. Disulfides, such as oxidized glutathione, react to form mixed disulfides, i.e. $E-SH + GSSG \rightleftarrows E-SSG + GSH$. Oxidized glutathione has a net charge of -2, and the addition of what amounts to "half" an oxidized glutathione molecule to the enzyme alters the net charge on the latter by -1 per reactive $-SH$ group. Other parameters being equal, a change in electrophoretic mobility will be the result. Alkylating agents, such as iodoacetamide, iodoacetic acid, or NEM, constitute a second group of sulfhydryl reagents, and these form a stable adduct with the protein. Some of these reagents alter the electrophoretic mobility, e.g. iodoacetic acid (net charge = -1), while others do not (e.g. NEM). Organic mercurials, e.g. pCMB, react to form mercaptides, and pCMB does alter electrophoretic mobility (it has a net charge of -1). Through systematic studies with such reagents, data were obtained that indicated a single sulfhydryl group for each ADA enzyme. The "storage effects" are due to the formation of mixed disulfides between the enzyme and oxidized glutathione, which accumulates in stored hemolysates. Electrophoresis is, therefore, generally carried out on samples to which 10 mM mercaptoethanol has been added. This subject has been well discussed by Hopkinson (1975) in terms of a number of polymorphic red cell isoenzymes, including ADA.

Osborne and Spencer (1973) partially purified ADA from hemolysates of the three main phenotypes. The isoenzymes of each type were partially resolved by chromatography on DEAE Sephadex. The MW of the isozymes, as estimated by gel filtration, was 30,000 to 35,000. The four components of ADA 2-1 had isoelectric points of 4.7, 4.83, 4.94 and 5.06 by isoelectric focusing. The K_m of the isozymes for adenosine was 30μM for all isozymes. Some differences in heat inactivation profiles were noted among the isozymes, and the heat inactivation characteristics showed a marked dependence upon ionic strength.

Daddona and Kelley (1977) purified red cell ADA some 800,000-fold. The preparation was homogeneous by antibody affinity chromatography, using an antibody developed against purified calf intestinal enzyme, which cross-reacted with the red cell one. The purified preparation showed three bands of ADA activity on polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single band with an apparent MW of 41,700. The MW of the purified protein as estimated by ultracentrifugation studies was 38,000.

A broad pH optimum from 7 to 8 was observed. The K_m for adenosine was 52 μM, and the K_i for inosine was 700 μM. At 4°, the preparation retained activity for up to 3 weeks, but at $-70°$, glycerol or DMSO was necessary to preserve activity during freezing-thawing cycles. The purified enzyme consisted of a single polypeptide chain, and staining of polyacrylamide gels with periodic acid-Schiff reagent indicated the presence of carbohydrate. The differences in isoenzyme structure responsible for the molecular heterogentity were not established by this study.

A method for the purification of ADA from human erythrocytes was given by Agarwal and Parks (1978).

The metabolic role of ADA is not yet completely clear. Interest in blood ADA was stimulated by the suggestion of Berne (1964) that adenosine (and its phosphorylated derivatives) might play a regulatory role in coronary blood flow because of their vasodilator properties. Blood adenosine

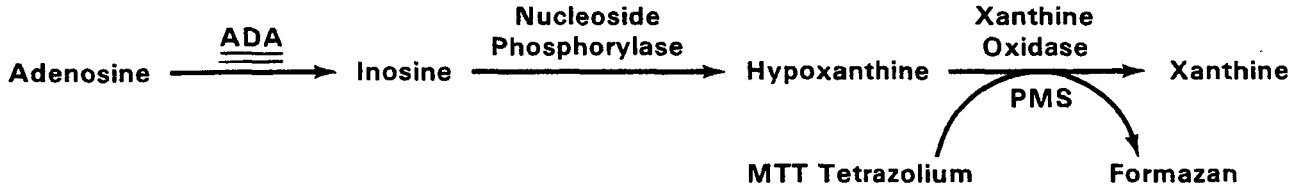


Figure 30.3 Detection Reaction Sequence for ADA

concentration is obviously influenced by the activity of ADA. Van Belle (1969) found that adenosine deamination in circulating blood was temperature dependent, but not affected by pH in the 6 to 8 range, nor by ionic strength. The situation was different in other animal species. More recently, interest in ADA has been prompted by the association between a deficiency of the enzyme and severe combined immuno-deficiency disease in certain patients (see section 30.2). Perrett and Dean (1977) have studied adenosine (and adenine) metabolism in human red cells over a range of substrate concentrations. At physiological adenosine concentrations, less than 1 μM, less than 10% of the adenosine was acted upon by ADA. Most of it was converted to adenine nucleotides by adenosine kinase. At much higher adenosine concentrations, in excess of 7 μM, deamination was the dominant pathway. If these findings accurately reflect the *in vivo* situation, then the role of ADA in purine metabolism is minimal.

30.5 Medicolegal Applications

30.5.1 Disputed parentage

ADA phenotyping is employed in a number of laboratories in cases of disputed parentage. Wüst (1971b) discussed the application of ADA typing to these cases, and said that about 5.5% of falsely accused men would be excluded by ADA phenotyping alone in the Viennese population. Bauer and Herbich (1972) recommended incorporation of the system into paternity investigations as well. They said that about 4% of falsely accused men would be excluded by the system in the Germanic population which they studied. Lefèvre *et al.* (1972) gave a detailed discussion of the probability calculations for paternity cases using the ADA system. Boorman *et al.* (1977) noted that 4.5% of falsely accused English men would be excluded by ADA phenotyping. Polesky *et al.* (1976) gave figures of 4.5% for Caucasians and 1.9% for Blacks for the overall probabilities of excluding a true nonfather in the U.S. population. ADA isozyme patterns are fully developed in fetal blood (Chen *et al.*, 1977). Caution should be exercised in the interpretation of second order exclusions based on ADA, since silent alleles are known to occur rarely.

30.5.2 ADA phenotyping in dried bloodstains

ADA phenotyping in bloodstains was described by Culliford in 1971. The buffer system originally described by Spencer *et al.* (1968), consisting of 0.1M phosphate, pH 6.5, bridge buffer, and a 1:10 dilution of that solution for gel buffer, was used. Electrophoresis was carried out on thin, 10% starch gels. Samples were treated with 1% mercaptoethanol in gel buffer prior to electrophoresis to insure that sulfhydryl effects (see in section 30.4.2) would not interfere with the interpretation of phenotypic patterns. ADA could be typed in 3 to 4 week old stains without difficulty, and occasionally in stains up to 3 months old. In samples infected with bacteria, activity could be lost, but in some samples of this kind, a single band occurred at a position cathodal to (slower than) the *ADA*^{2} band.

Brinkmann and Dirks (1971) described ADA typing in bloodstains as well. Electrophoresis was carried out a system which allowed simultaneous determination of ADA, AK and PGD. ADA 1 and ADA 2-1 phenotypes could be distinguished in bloodstains up to 5 months old, but ADA 2-1 and ADA 2 were difficult to distinguish in the older stains.

Welch (1972b) found that ADA was detectable in 15 day old stains but not in 22 day old ones. Denault *et al.* (1978) reported that ADA was detectable in 13 week old stains on a variety of substrata, regardless of whether the phenotype was 1 or 2-1. Bloodstains kept at higher humidity gave better results than those kept at low humidity. At 26 weeks of age, only one of 14 stains was able to be phenotyped, and this stain had been kept frozen.

A number of variations in electrophoretic procedure have been applied to ADA phenotyping. Bauer and Herbich (1972) described a high voltage procedure for hemolysates on starch gels which was said to reduce running time and give sharper patterns. Sonneborn and Renninger (1970) described a cellulose acetate membrane procedure for ADA typing in hemolysates. The technique was further discussed by Sonneborn in 1972. Hoppe *et al.* (1972) gave a horizontal polyacrylamide gel technique for ADA.

A number of techniques have been described in which ADA is simultaneously determined with other isozyme systems in the same electrophoretic operation: ADA with AK on starch gels (Hummel, 1970; Kirchberg and Wendt, 1970); ADA with AK, ACP and PGM on one thick starch gel (Martin and Niebuhr, 1971); ADA with PGM and AK on horizontal polyacrylamide gels (Wrede *et al.*, 1971); and ADA with ACP and AK on starch gels in a system designed for bloodstain phenotyping (Wraxall and Stolorow, 1978).

30.5.3 ADA phenotyping in other tissues

ADA occurs in a variety of tissues apart from red blood cells, as indicated in the discussion in section 30.3. The evidence also suggests that a single genetic locus is responsible for all the red cell and tissue isozymes observed. Oepen (1974) found that ADA could be determined in fresh, dried or refrigerated skeletal muscle tissues, but that it was considerably more labile than the AK enzymes in this material. Turowska and Trela (1977) described ADA detection in the tissues of human teeth. ADA does not occur in detectable amounts in either seminal plasma or spermatozoa (Blake, 1976; Blake and Sensabaugh, 1976).

30.6 Distribution of ADA Phenotypes in U.S. Populations

There are only a few published studies of ADA phenotype distributions in this country, and the data are shown in Table 30.1.

The data of van den Branden *et al.* (1971) covered a number of different populations in other countries. The *ADA*[1] frequency in Europeans varies from about 0.91 to 0.95. In Black African peoples, it is somewhat higher. The only populations studied in which *ADA*[2] gene frequencies are relatively high are Asians and the Kurds of Iran, where the frequency can exceed 0.1 (van den Branden *et al.*, 1971; Mourant *et al.*, 1976).

Table 30.1 Distribution of ADA Phenotypes in U.S. Populations

		Frequency — Number (Percent)					
Population	Total	1	2-1	2	Other	*ADA*[1]★	Reference
CAUCASIAN							
Seattle, WA	168	152 (90.5)	16 (9.5)	0		0.950	Detter et al., 1970
Philadelphia, PA	180	(88.1)	(11.9)	(0)		–	Polesky et al., 1976
California and Hawaii	5,883	(90.0)	(9.8)	(0.2)	(0)	0.949	Grunbaum et al., 1978b
Miami/Dade County, FL	360	323 (89.7)	36 (10.0)	1 (0.3)		0.947	Stuver, 1979 and see Shaler, (1978)
Detroit, MI	503	446 (88.7)	56 (11.1)	1 (0.2)		0.942	Stolorow et al., 1979 and see Shaler, (1978)
Los Angeles, CA	135	123 (91.1)	12 (8.9)	0		0.956	Siglar, 1979 and see Shaler, (1978)
NEGRO							
Seattle, WA	186	178 (95.7)	6 (3.2)	0	two "5-1"	0.980	Detter et al., 1970
Philadelphia, PA	180	(97.2)	(2.8)	(0)		–	Polesky et al., 1976
California and Hawaii	927	(97.8)	(2.2)	(0)	(0)	0.989	Grunbaum et al., 1978b
Miami/Dade County, FL	344	333 (96.8)	11 (3.2)	0		0.984	Stuver, 1979 and see Shaler, (1978)
Detroit, MI	504	496 (98.4)	8 (1.6)	0		0.992	Stolorow et al., 1979 and see Shaler, (1978)
Los Angeles, CA	56	54 (96.4)	2 (3.6)	0		0.982	Siglar, 1979 and see Shaler, (1978)
HISPANIC							
California and Hawaii	1,260 ☆	(93.8)	(5.9)	(0.3)	(0)	0.964	Grunbaum et al., 1978b
Miami/Dade County, FL	355	329 (92.7)	24 (6.7)	2 (0.6)		0.961	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA	81 ⊙	77 (95.1)	4 (4.9)	0		0.975	Siglar, 1979 and see Shaler, (1978)
ASIAN/ORIENTAL							
Seattle, WA "Mixed Oriental"	118	113 (95.8)	5 (4.2)	0		0.980	Detter et al., 1970
California and Hawaii "Asian"	1,821	(95.2)	(4.6)	(0.2)	(0)	0.975	Grunbaum et al., 1978b

★ Gene frequency ☆ "Chicano/Amerindian" ⊙ Primarily Mexican

SECTION 31. ESTERASES

31.1 Introduction to Esterases

There are a number of carboxylic ester hydrolases in blood and in tissues, each group of enzymes possessing its own properties and peculiarities. Not all of these groups show genetic variation. The ones which do not are discussed only briefly in the following sections for the sake of completeness.

Generally, enzymes which exhibit esterase activity may be divided into three major categories: carbonic anhydrases; cholinesterases; and carboxylesterases. Carbonic anhydrases, which exhibit esterase activity, will be discussed in section 32. This section is concerned with cholinesterases and carboxylesterases. Many esterases exhibit very broad substrate specificities. In addition, there are many different esterase isoenzymes, and the physiological significance of many of them is not clear. For these reasons, attempts to classify the esterases, and to understand and account for them from a genetic point of view, have met with difficulty.

31.2 Cholinesterases

31.2.1 Recognition and classification of cholinesterases

In 1914, Dale suggested that a mechanism for the very rapid hydrolysis of acetylcholine might exist in the body, in order to remove this substance after it had served its purpose as a neurohumoral transmitter. Indeed, it is now known that acetylcholinesterase occurs in the nervous tissue of all animals. In 1932, Stedman *et al.* established that horse serum contained a cholinesterase activity. Glick (1937) carried out studies of the enzyme in human serum. In 1940, Alles and Hawes established that human blood contains two different sorts of cholinesterase activity, one being found in serum, while the other occurred primarily in red cells. These two activities differed significantly in their properties. Numerous studies have been conducted on both enzymes over the years.

The cholinesterases of human blood are thus of two kinds: (1) the cholinesterase found in red cells, which is called acetylcholinesterase (AChE; E.C. 3.1.1.7; true cholinesterase; acetylcholine hydrolase); and (2) the cholinesterases of plasma (or serum), which do not hydrolyze acetylcholine preferentially, and have been given a variety of different names, including pseudocholinesterases (E.C. 3.1.1.8). Cholinesterases are characterized simply by the fact that they hydrolyze choline esters. In general, they may be distinguished from other kinds of esterases by virtue of the fact that they are inhibited by 10^{-5}M eserine (physostigmine). The cholinesterases are, however, a family of enzymes with many divergent properties (Augustinsson, 1957). Studies on the purification and properties of acetylcholinesterases from a number of sources have been reviewed by Wilson (1960), Koelle (1963) and Froede and Wilson (1971).

31.2.2 Red cell acetylcholinesterase (E.C. 3.1.1.7)

In 1962, Johns described a patient who had only about one-third the normal red cell AChE activity. The man was healthy. It was found that his mother and sister shared this reduced red cell AChE activity, strongly suggesting a genetic basis for the condition. The red cell enzyme is bound to, or perhaps an integral part of, the erythrocyte membrane, and, as with other membrane-associated proteins, special procedures must be employed to solubilize it. As a result, this enzyme does not readily lend itself to electrophoretic analysis. In 1972, Coates and Simpson reported that they had detected genetic variation in the enzyme by disc electrophoretic analysis of Triton-X-100-extracted red cell stroma. There were three apparent phenotypes, called 1, 2-1 and 2, and limited family studies indicated that these phenotypes were consistent with control of the enzyme by an allelic pair, called $AChE^1$ and $AChE^2$. In 1976, Das and Lo obtained somewhat similar results using a somewhat different polyacrylamide gel electrophoresis procedure. Additionally, their material was obtained exclusively from Chinese men. They could distinguish three patterns as well, and called them 1, 2 and 3. Type 2 looked quite similar to the Type 1 of Coates and Simpson (1972). They did not do family studies, however, and did not say explicitly that the observed variation had a genetic basis.

A number of studies have been carried out on the purification and characterization of red cell AChE, and it is not very clear from these investigations whether this enzyme does or does not really show genetic variation. In 1971, Shafai and Cortner purified red cell AChE to a certain extent, and could resolve two components on DEAE-Sephadex which differed in charge, but were similar in size (Shafai and Cortner, 1971a). Further studies on DEAE ion exchange media suggested that the enzyme consisted of subunits, which could dissociate and reassociate on the column itself, during the separation (Shafai and Cortner, 1971b). Ciliv and Özand (1972) purified the enzyme, and found it to be a glycoprotein which formed easily reversible aggregates. Paniker *et al.* (1973) studied the effects of using a number of non-ionic detergents in the purification of the enzyme, and there were no differences, suggesting that membrane dissolution was the key requirement in releasing the enzyme from the stroma. Wright and Plummer (1973), Das and Lo (1976) and Das *et al.* (1977) have all reported purification of the enzyme, and all have observed apparent multiple forms of it on ion-exchange chromatographic media and/or disc electrophoresis. There is not much doubt that some of the different forms represent different states of aggregation

of the same subunit(s). The purest preparations have been obtained by affinity chromatography of Triton-X-100-solubilized membrane fractions (Grossmann and Liefländer, 1975; Ott *et al.*, 1975; Ott and Brodbeck, 1978). Studies on the pure preparations indicate that the enzyme behaves as a single molecule with MW 80,000 in the presence of solubilizing detergent, but that the detergent-free enzyme shows multiple forms upon ion exchange media, polyacrylamide gels, isoelectric focusing, or density gradient centrifugation. The multiple forms are presumably different aggregates of the 80,000 MW subunit. Grossmann and Liefländer (1975) said that the MW of the purified preparation, as estimated on detergent (SDS)-containing polyacrylamide gels was 80,000 in the presence of mercaptoethanol but 154,000 in its absence, suggesting a disulfide bond in the molecule.

31.2.3 Plasma (Serum) cholinesterase (ChE; Pseudocholinesterase; PCE; E.C. 3.1.1.8; acylcholine acyl-hydrolase)

31.2.3.1 Early studies on plasma ChE. The studies leading to the recognition of plasma ChE enzymes, with an identity separate from the red cell cholinesterase, were mentioned in section 31.2.1. The earlier studies on plasma ChE have been reviewed by Augustinsson (1948 and 1961) and the same author has written an extensive review of the assay methods employed for ChE (Augustinsson, 1957).

31.2.3.2 Genetically controlled variation in plasma ChE. Interest in plasma ChE was stimulated in part because of the role of this enzyme in hydrolyzing the muscle relaxant succinyldicholine (also called suxamethonium; Fig. 31.1). The drug was apparently quite commonly used in patients undergoing anesthesia. It was known to be short-acting, because of its rapid hydrolysis by serum ChE. In 1956, Kalow established with certainty, both *in vitro* and *in vivo*, that plasma ChE hydrolyzes succinyldicholine, and he carried out measurements on the reaction (Kalow, 1956a). Three people were described, however, whose serum showed a very low succinyldicholinesterase activity. They had been detected because of adverse reactions to the usual dosages of the drug. Profound muscle relaxation followed administration of the substance in these people, along with a period of apnea (cessation of respiration). Many people with unusual kinds of plasma ChE have been detected through their response to the routine administration of succinyldicholine in clinical settings. Goedde *et al.* (1968) suggested that pro-

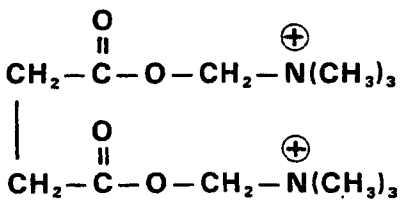


Figure 31.1 Succinyldicholine

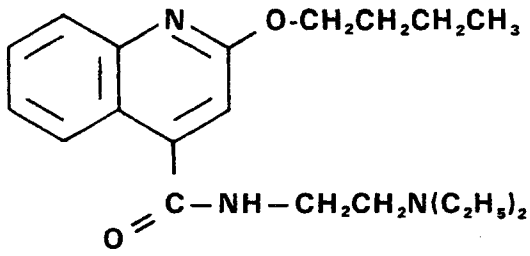


Figure 31.2 Dibucaine

longed apnea following treatment with succinyldicholine be treated by administration of purified plasma ChE.

Kalow and Genest (1957) soon showed that the unusual ChE present in the sera of the suxamethonium-sensitive people could be detected in another way, namely on the basis of inhibition of the enzyme by dibucaine (Fig. 31.2). Dibucaine is a local anaesthetic, and is also called Nupercaine, Percaine and cinchocaine. Using benzoyl choline as substrate for the enzyme, assays were conducted with and without 10^{-5}M dibucaine. Under a standard set of conditions, 5×10^{-5}M substrate in 66.67 mM phosphate buffer, pH 7.4, and 25°, serum was assayed at a 1:100 dilution by following the decrease in absorbance at 240 nm. Normal (usual) sera were inhibited about 79%, but the "atypical" sera only about 16%. The % inhibition with dibucaine seen under these standard conditions was defined as the "Dibucaine Number" (or DN). In 1957, Kalow and Staron reported extensive cholinesterase assay studies on almost 1,700 sera, many from members of families. Using the DN, three different groups could be distinguished, and these were called "usual", "intermediate" and "atypical". Family studies indicated that these phenotypes could be accounted for on the basis of a pair of codominant alleles operating at an autosomal locus. The "usual" allele and the "atypical" allele were presumably responsible for different cholinesterase enzyme molecules, which were found in approximately equal mixture in the plasma of heterozygotes ("intermediate" types). The frequency of the atypical gene in the Toronto area was about 0.015. Kalow and Davies, in 1959, tested a series of esterase inhibitors for their ability to discriminate the three phenotypes. Most showed a differential effect on the usual and atypical forms, like dibucaine does. The organophosphates (such as DFP), however, which are classical inhibitors of ChE, inhibited both forms equally. These inhibitor studies were consistent with the genetic hypothesis. Harris and Whittaker (1962b) carried out family studies on the serum ChE polymorphism, and the results were fully consistent with the two allele hypothesis.

Other ChE inhibitors, as noted above, will differentially inhibit the usual and atypical forms of ChE, and one interesting inhibitor was first found in potatoes. In 1956, Pokrovskiy observed that watery extracts of potatoes would inhibit plasma ChE. Orgell *et al.* (1958) confirmed this finding, and showed further that many plants which were

members of the *Solanaceae* contained the inhibitor. The potato (*Solanum tuberosum* L.), which has the inhibitor in the tuber and the tuber peel, was especially well studied. The results indicated that the inhibitor was not a macromolecule. Harris and Whittaker (1959) showed that the potato extract inhibitor differentially affected the usual and atypical serum ChE enzymes, and could therefore be used to discern the phenotypes. Harris and Whittaker (1962a) carried the studies further. They gave credit to Pokrovskiy (1956), whose work they had not known about, for establishing that the potato inhibitor was solanine, and they showed that solanine, and its alkaloid moiety, solanidine (Fig. 31.3), both give the differential inhibition of plasma ChE types.

31.2.3.3 Further genetic variation in plasma ChE. In 1961, Harris and Whittaker examined the plasma ChE of all three "dibucaine" types for inhibition by 5×10^{-5}M NaF. In general, sera fell into three categories based upon inhibition by NaF, and these corresponded to the division seen with dibucaine as inhibitor. Fluoride Number (FN) was defined in the same way as Dibucaine Number, except for the difference in inhibitor concentration. DN values for the three phenotypes are about 80, 62 and 20 for usual, intermediate and atypical forms, respectively, and the corresponding FN were 61, 48 and 23. Some discrepancies were found, however, in which the FN was about 26–34 (with DN about 52–54), and in which FN was 50–55 (with DN 73–78). The values were consistent over time, and were attributed to the expression of another allelic gene at the ChE locus, in heterozygous combination with one of the known alleles. Family studies by Harris and Whittaker (1962b) supported this notion.

In 1968, Whittaker found that aliphatic alcohols would differentially inhibit the usual and atypical ChE enzymes. The effects were rather complicated, but alcohol concentrations and other reaction conditions could be found where the differential inhibition was maximal (Whittaker, 1968a). Further studies (Whittaker, 1968b) were carried out with n-butanol. An "Alcohol Number" was defined in the same way as had been the dibucaine and fluoride numbers (i.e., as % inhibition). The numbers were different from the DN or FN for similar types, but generally followed the same pattern. There was a suggestion that alcohol inhibition studies might define still more phenotypes of ChE. Whittaker (1968c) also found that NaCl (0.5M) would differentially inhibit the usual and atypical enzymes, and these values of inhibition were called "Chloride Numbers". The work of Clark *et al.* (1968), discussed in section 31.2.3.6, might be relevant to these NaCl inhibition studies.

In 1966, Neitlich described a man with consistently elevated plasma ChE, and studies on his family indicated that the condition had a genetic basis. Yoshida and Motulsky (1969) studied the ChE from this individual by immunological and biochemical techniques, and concluded that a structurally abnormal enzyme was present, and was being "overproduced". This variant type was called "Cynthiana".

31.2.3.4 Electrophoretically detectable genetic variation in plasma ChE—a second plasma ChE locus. In 1962, Harris *et al.* carried out an electrophoretic study of plasma ChE, using two dimensional electrophoresis. Serum was run first on paper, and the paper was then inserted into a starch gel, and electrophoresis carried out at right angles to the direction of the first separation. Electrophoretic steps were done in pH 7.1 phosphate buffers, and the ChE detected with α-naphthyl acetate and Fast Red TR salt. In most sera, four zones could be distinguished. These were designated C_1

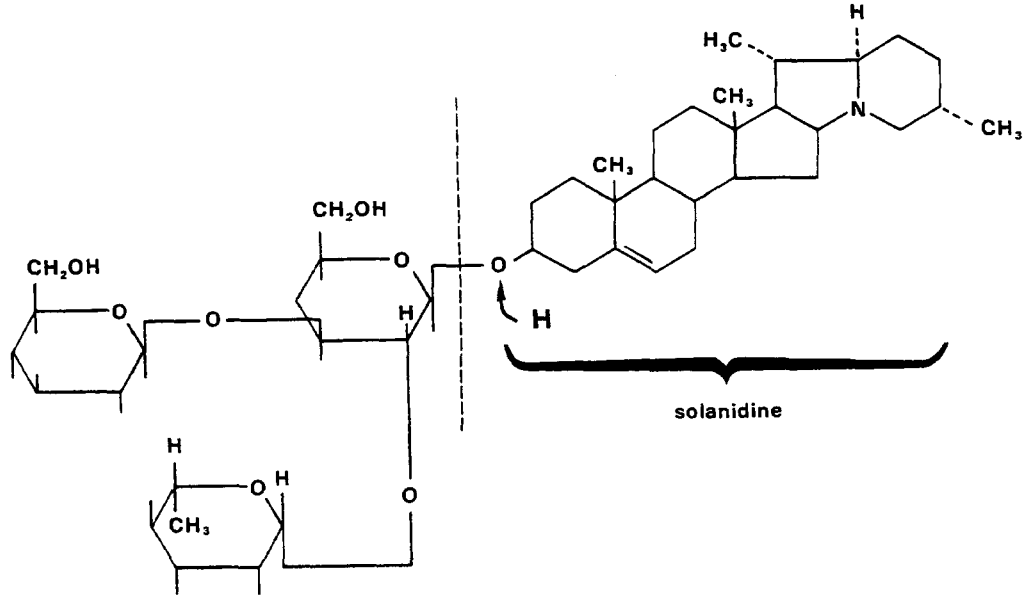


Figure 31.3 Solanine

through C_4, the last representing most of the enzyme activity. Occasionally, a fifth zone was seen. It was less anodal (slower), and closer to the origin, than C_4, the slowest of the other four common isozymes, and it was called C_5. Further studies by Harris *et al.* (1963a) supported a genetic basis for the presence of C_5 in some people's sera. About 5% of 248 British people were C_5^+. It was shown, too, that one dimensional starch gel electrophoresis at pH 6 would serve to distinguish between C_5^+ and C_5^- people. The C_5^+ phenotype was regarded as the expression of a gene whose allele did not code for the enzyme. Accordingly, no C_5^+ offspring should result from $C_5^- \times C_5^-$ matings, and, in a few cases, C_5^- parents with a C_5^+ child were seen in the study. Electrophoretic typing of C_5^+, however, is not always completely unambiguous. Difficulty in phenotyping and/or variability in the expression of C_5^+ in C_5^+ individuals could explain the apparent discrepancies in the family material. Ashton and Simpson (1966) raised this problem as well, noting that there had been several discrepancies in the typing results carried out by different laboratories on the same sera. They noted that caution was necessary in the interpretation of the C_5 phenotypes. There were some significant deviations from the expected segregation patterns in 1,000 Brazilian families in these studies.

Harris *et al.* (1963b) studied the expression of the "dibucaine sensitivity" and of C_5^+ simultaneously, and the data indicated quite conclusively that the loci for the two types of plasma cholinesterases were not identical. Further studies by Robson and Harris (1966) supported the notion of a separate locus for the gene controlling the C_5^+ polymorphism, and they noted that the C_5 isoenzyme might not always be detected in heterozygotes by standard electrophoretic typing procedures.

By 1964, some general agreement had been reached among workers in the field on plasma ChE nomenclature. The recommended scheme was summarized by Motulsky (1964), and is given in Table 31.1. There are two known plasma ChE loci, called E_1 and E_2. Four alleles are known to occur at E_1, and they are denoted by superscript lower case letters: E_1^u, E_1^a, E_1^f, and E_1^s. The first three of these alleles control the usual, atypical and fluoride-resistant forms of plasma ChE, and E_1^s is silent (or almost silent; see section 31.2.3.5). There are two alleles at E_2, simply denoted E_2^+ and E_2^-, which give rise to three genotypes, $E_2^+E_2^+$, $E_2^+E_2^-$ and $E_2^-E_2^-$. The first two of these are indistinguishable, both being C_5^+.

31.2.3.5 Silent alleles at E_1. In 1956, Kalow and Lindsay reported that four people out of about 1,000 they had studied in Canada showed very low plasma ChE activity, almost too low to be measured. Independently, Lehmann and Ryan (1956) described an English family, a number of whose members had very low plasma ChE activity. Kalow (1956b) said that these observations were essentially in agreement with his own. The people were detected because

Table 31.1 Nomenclature and Properties of the Plasma Cholinesterase Variants

Genotype		Phenotype				Typical	
Standard Designation ★	Other Designation ☆	Standard Designation ★	Other Designation ☆	Type of Esterase Present	Amount of Esterase Present (Relative %)	DN ○	FN ◇
$E_1^u\ E_1^u$	N–N	U	usual	u(sual)	100	80	64
$E_1^u\ E_1^a$	N–D	I	intermediate	u + a(typical)	78	62	48
$E_1^a\ E_1^a$	D–D	A	atypical	a	25	20	23
$E_1^s\ E_1^u$	S–N	U	usual	u	65	80	64
$E_1^s\ E_1^s$	S–S	S	silent; zero	none	0	–	–
$E_1^s\ E_1^a$	S–D	A	atypical	a	20	20	23
$E_1^f\ E_1^u$	F–N	UF	U_1	f(luoride) resistant + u	80	76	52
$E_1^f\ E_1^f$	F–F	F		f	50	67	34
$E_1^f\ E_1^a$	F–D	IF	I_1	f + a	60	50	30
$E_1^f\ E_1^s$	F–S	F		f	never observed		
$E_2^+\ E_2^+$ } $E_2^+\ E_2^-$ }		C_5^+		u + C_5	130	80	64
$E_2^-\ E_2^-$		C_5^-		u	100	80	64

★ After Motulsky (1964) ☆ After Lehmann and Liddell (1964) ○ Dibucaine Number ◇ Fluoride Number

they were extraordinarily sensitive to succinyldicholine administration. In 1962, Liddell *et al.* studied a number of individuals who had shown unusual succinyldicholine sensitivity. They found subjects in whom the DN corresponded to homozygosity for E_1^a, but where family studies indicated heterozygosity for E_1^a. The best explanation of the results, they said, was heterozygosity of E_1^a with a silent allele at E_1. They described a Greek woman who had no plasma ChE, and thought that she was probably a homozygote for the silent gene.

Doenicke *et al.* (1963) reported on an individual with no detectable plasma ChE, and they confirmed the absence of activity in a biopsied liver specimen. In 1964, Simpson and Kalow agreed with Liddell *et al.* (1962) that absence of serum ChE was attributable to homozygosity for a silent allele at E_1, and they called it E_1^s. Although a suppressor gene could not be excluded, it has generally been accepted since that time that the $E_1^sE_1^s$ genotype is the correct explanation for an absence of plasma ChE activity. The frequency of E_1^s was estimated to be about 10^{-5}. Other cases of apparent homozygotes of E_1^s have been reported by Dietz *et al.* (1965), Hodgkin *et al.* (1965) in an American Irish family, Szeinberg *et al.* (1966) in a Morrocan Jewish family, and Jenkins *et al.* (1967) in an African Bantu girl.

There is considerable evidence that there is more to the "silent allele" story than was first realized. It appears now that not every "silent" serum is the same, and that there may be more than one genetic basis for the absence, or near absence, of plasma ChE activity. Goedde *et al.* (1965a and 1965b) examined sera from two people who were presumed to be homozygous for the silent allele. Upon close examination, the sera revealed low activity, 2-3% of normal. These sera also reacted with a precipitating antibody against normal plasma ChE prepared in rabbits, suggesting that, at least in these people, the "silent" gene coded for a structurally altered protein. These studies have been pursued by Goedde and Altland (1968) and Altland and Goedde (1970). On the basis of activity with different substrates, electrophoretic and chromatographic studies, and of the behavior of the "silent" sera toward anti-plasma ChE, it is clear that not every example of ChE-deficient serum has the same properties. These findings have been confirmed by other workers (Gaffney and Lehmann, 1969; Rubinstein *et al.*, 1970). There are cases in which the plasma has no activity whatsoever, apparently the result of a truly silent allele. Hodgkin *et al.* (1965) showed that the sera of their propositi did not react with an anti-normal ChE antibody, suggesting that no gene product was present. In general, ChE deficiency is extremely rare. If the estimate of Simpson and Kalow (1964) of 10^{-5} for the E_1^s frequency were correct, one would expect to find homozygosity for this allele in only about 1 in every 10 billion people. From the published results to date, more people than that have been found, but the frequency is still very low. The Eskimos of Western Alaska have been shown to have an inordinately high frequency of plasma ChE deficiency (Gutsche *et al.*, 1967; Scott, 1973). 37 people out of 5,000 have been found to be deficient. There are some people with complete deficiency, while others have a trace of activity, and it was suspected but not proven that the two conditions have a different genetic basis.

31.2.3.6 Molecular heterogeneity of plasma ChE—Biochemical studies. Over the years, a considerable number of studies have indicated that plasma ChE shows molecular heterogeneity, and that a number of isozymes can be identified, apart from the C_5 polymorphism. There was early kinetic evidence that serum contained more than one ChE activity (Heilbronn, 1958; Berry, 1960). Chromatography of plasma on calcium phosphate gels gave similar indications (Malmström *et al.*, 1956). Many investigators have examined plasma ChE isozymes by electrophoresis (Dubbs *et al.*, 1960; Bernsohn *et al.*, 1961; Hess *et al.*, 1963; Brody *et al.*, 1965; Juul, 1968). Depending upon the technique used, anywhere from 2 to 12 zones of ChE activity have been detected. On starch gel electrophoresis, it is common to observe 4 zones of activity, usually called C_1 through C_4. C_4 accounts for the majority of the total activity. These electrophoretically distinguishable forms can also be separated by gel filtration chromatography (Harris and Robson, 1963b), and the C_5 isozyme has been fractionated on calcium phosphate gels (Reys and Yoshida, 1971).

Svensmark (1961a and 1961b) established that plasma ChE was a sialoprotein, and the change in electrophoretic mobility following treatment with neuraminidase suggested that the various forms contain a different number of sialic acid residues per molecule. Removal of the sialic acid with neuraminidase did not affect enzyme activity though. A number of workers have purified the enzyme to a greater or lesser degree, and examined the properties of the multiple molecular forms. Svensmark (1965) partially purified plasma ChE, and estimated that the MW of the major fraction was 300,000. There was no indication of a prosthetic group, and no metal requirement. The atypical enzyme was found to differ from the usual one in substrate and inhibition characteristics. LaMotta *et al.* (1965, 1968 and 1970) purified the enzyme, and found that the isoenzymic forms were interconvertible. In some purified preparations, up to 7 isozymes could be detected. Based upon MW estimates for the major isozyme, they suggested that the different forms might simply be polymers of a subunit with MW about 30,000. Gaffney (1970), who observed six zones of activity in purified preparations by polyacrylamide gel electrophoresis, agreed that the forms differed in size (as well as in charge). He did not think, however, that the simple polymerization hypothesis of LaMotta *et al.* (1970) sufficed to explain his data. The interconvertibility of ChE forms might help to explain the observation by Dubbs (1966) that sonication in serum for about 15 min. greatly enhanced the intensity of the C_3 zone. Haupt *et al.* (1966) obtained a very pure preparation of plasma ChE, and the MW was said to be 348,000. They also noted that serum ordinarily contains about 0.9 mg of the enzyme per 100 mℓ of serum. Muensch *et al.* (1976) prepared a very pure C_4 (about 8000-fold). It had a MW of 345,000 and was found to be a tetramer of C_1.

These studies did not shed light on the nature of C_2 or C_3, nor on the role of the sialic acid residues in the isozymes.

In connection with the studies on C_5 (E_2) phenotyping, there have been several reports of additional bands less anodal than C_5 in fresh serum samples. Ashton and Simpson (1966) observed an additional band, a kind of "C_6". Van Ros and Druet (1966) observed a C_6 and two additional bands, C_{7a} and C_{7b}, in two Black African individuals, and suggested that these were genetically determined, although family studies could not be done. Ogita (1975) came up with a rather different interpretation of these patterns based on his studies of several Japanese subjects with unusual plasma ChE. The C_6 and C_7 forms of plasma ChE appeared in the serum of an old man who had a benign peritoneal tumor. His plasma ChE level had dropped to very low levels during the course of the disease, but later increased somewhat. Incubation of his serum with ordinary C_4 ChE induced the formation of the C_6 and C_7 components, and this transformation was shown to be due to a neuraminidase-like activity in his serum. There were indications from the family study that the neuraminidase-like activity was under genetic control, and Ogita suggested that some of the ChE components occasionally observed in plasma may have nothing to do with the E_1 or E_2 loci directly, but might represent epigenetic modifications.

Clark *et al.* (1968) carried out extensive kinetic studies on plasma ChE and on partially purified C_4 component. These experiments compared the properties of the usual and the atypical enzyme. Tris buffer was found to have complex stimulatory and inhibitory effects on both types of enzymes. At low concentrations of Tris, about 0.67 mM, NaCl in increasing concentrations enhanced the activity of the usual enzyme but not of the atypical one. Other salts had similar effects. Tris itself, at higher concentrations, stimulated both forms of the enzyme. The usual one was stimulated more, and the kinetics were complex. These effects were seen with benzoylcholine as substrate, but not when acetyl- or butyrylthiocholine were employed. These data, together with that from other series of experiments, led to the suggestion that the alteration in the atypical, as compared with the usual, enzyme affected both the esteratic and anionic sites in the molecule. Cholinesterases are thought to have both kinds of sites. The anionic site is a locus of negative charge, and attracts the quaternary ammonium group of the choline. This site determines the specificity of the enzyme with respect to the alcohol moiety of the ester. The esteratic site is the actual catalytic site, and determines the specificity of the enzyme toward the acid moiety of the ester (see Froede and Wilson, 1971, for review of the catalytic mechanism of AChE). The studies of Lockridge and La Du (1978) on highly purified usual and atypical ChE enzymes with a fluorescent probe substrate indicate that the two differ only in the structure of their anionic site. Lockridge *et al.* (1979) have extensively purified the usual ChE enzyme and studied its subunit organization. The native molecule is a tetramer of MW 340,000, and appears to be arranged as a dimer of dimers. Each dimer contained one interchain (and several intrachain) disulfide bonds. The subunits are apparently held together by noncovalent forces.

Reviews covering the plasma ChE variants may be found in Lehmann and Liddell (1964) and Simpson (1968).

31.2.3.7 Assay methods for ChE—Detection of E_1 phenotype and screening techniques. There are many different assay techniques for serum ChE. If phenotyping is the objective of the measurements, however, then standard assay techniques must be employed, since the E_1 phenotypes represent quantitative differences in enzyme activity in the presence and absence of specific concentrations of specific inhibitors. Assay methods have to be standardized and uniform so that results from different laboratories will be comparable. The original, and standard, method for the determination of Dibucaine Number (DN) was given by Kalow and Genest (1957). The method for the determination of Fluoride Number (FN) is the same, except that 5×10^{-5}M NaF is used instead of 10^{-5}M dibucaine (Harris and Whittaker, 1961). Any other procedure would have to be carefully standardized with established examples of sera of known phenotype.

If a large number of sera are to be tested, then spectrophotometric assay procedures are time consuming and cumbersome, and investigators have sought more rapid tests for the initial screening of sera. The point of such tests is the detection of possible variants, which may then be subjected to the more rigorous assay. In 1962, Kalow described a screening procedure which was relatively quick,, but which was spectrophotometric. Kalow and Davies (1959) had discovered that an inhibitor, called RO2-0683, at 10^{-7}M, completely inhibited the usual enzyme, but inhibited the atypical enzyme by about 30%, and "intermediate" serum activity by about 60%. This inhibitor was employed in the screening test at 2.26×10^{-6}M.

RO2-0683 was an experimental substance from Roche, and is chemically the dimethyl carbamate of 2-hydroxy-5-phenyl-benzyl trimethylammonium bromide (Fig. 31.4). It is first referred to as "RO2-0683" by Kalow and Davies (1959). The compound was previously called "Nu 683", and was introduced by Hawkins and Gunter (1946), who found that it was a selective inhibitor of plasma ChE. Myers (1952) did extensive studies on the characteristics of the inhibition of plasma ChE by the compound. Most screening tests have employed RO2-0683 as inhibitor.

In 1963, Harris and Robson described two types of screening tests for atypical and intermediate ChE phenotypes (Harris and Robson, 1963a). One of these was carried out in 1.5% agar gel, prepared in 0.1M Tris HCl, pH 7.4. Two identical gel plates were prepared, one containing 10^{-7}M RO2-0683, and the other not containing it. Identical sets of wells were punched in the gels, and the same serum was added to the corresponding wells of each plate. Serum dilutions were 1:32 for the control gel, and 1:8 for the inhibitor gel. After 17 hours of diffusion, the gels were flooded with α-naphthyl acetate in phosphate buffer, pH 7.1, containing Fast Red TR salt. Sera of the usual type showed activity in the control gel well, but no activity in the inhibi-

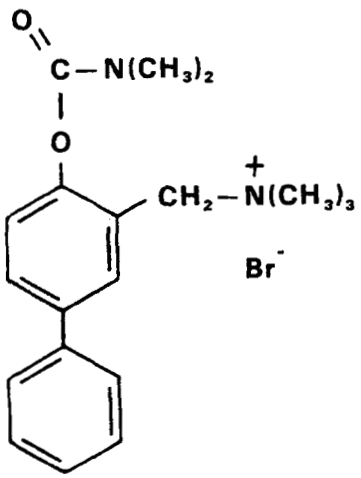


Figure 31.4 RO2 - 0683

tor gel well. Sera which showed activity in both were put aside as presumptive intermediate or atypical types, and subjected to spectrophotometric assay. The other procedure was done in a similar way, but in solution in test tubes. Drops of the solution were then applied to filter paper sheets containing substrate and coupling azo dye. Harris and Robson (1963a) tried the screening test on a number of sera of known type and it performed accurately. Goedde and Fuss (1964) described an agar gel screening test that was quite similar. Radam (1966) offered an improved filter paper screening test that was said to be more sensitive, primarily because a different azo coupling dye was employed. Simpson and Kalow (1965) used the agar gel screening test of Harris and Robson (1963a) to screen the sera of 6,500 people. There were some cases in which they said the screening test failed (0.1% of the sera). It also gave evidence of "atypical" sera in a number of cases where subsequent assay showed the serum to be of the "usual" phenotype.

The C_5^+ phenotype is detectable by electrophoresis, as noted above. A procedure is described by Harris and Hopkinson (1976) in addition to those given in the references cited in section 31.2.3.4. Scott and Weaver (1970) mentioned that electrophoresis could be used to detect simultaneously the C_5^+ variants and the low or zero activity ones. They noted that thiocholine, produced by the hydrolysis of butyrylthiocholine by ChE, will reduce MTT tetrazolium in the presence of PMS and DCPIP, yielding the familiar formazan bands of enzymatic activity.

31.3 Carboxylesterases

31.3.1 Classification of carboxylesterases

Carboxylesterases occur in a wide variety of tissues. Over the years, classification schemes have been proposed to bring some order to this diverse group of enzymes. The classifications are based primarily on substrate specificity of the enzymes, and on differential inhibition by various inhibitors, regardless of tissue of origin. In 1953, Aldridge designated two different forms of esterases in a number of animal sera as "A" and "B". A-esterases hydrolyzed p-nitrophenyl acetate better than p-nitrophenyl butyrate, and were not inhibited by diethyl p-nitrophenyl phosphate (called E-600); B-esterases hydrolyzed the butyrate ester as well as or better than the acetate ester, and were inhibited by about 10^{-7}M E-600. He found too that the A-esterase hydrolyzed the E-600 itself. In 1957, Bergmann *et al.* isolated an esterase from hog kidney whose properties were unlike either the A- or B-esterases, and they designated it a C-type esterase. The substrate specificity and inhibitor sensitivity characteristics of A and B esterases were discussed by Augustinsson (1961). Red cell esterases belonging to these three classes have been studied extensively (section 31.3.2) and the different classes of esterases can be separated electrophoretically, in addition to being distinguishable on the basis of substrate specificity and inhibitor properties. The same can be said for tissue esterases (section 31.3.6). The older carboxylesterase classification is shown in Table 31.2, and a more recent scheme appears in Table 31.3.

31.3.2 Red cell carboxylesterases

In 1958, Wachstein and Wolf showed by histochemical staining techniques that human red cells contained esterase activity with naphthol-AS-acetate. These results were confirmed by Davis (1959) using α-naphthyl acetate as substrate. As noted in section 26, esterases were among the first enzymes demonstrated to consist of multiple molecular forms by the combination of electrophoresis followed by histochemical staining of the separated enzymes. The esterase isozymes of mouse blood and tissues were extensively studied (Markert and Hunter, 1959; Hunter and Strachan, 1961). There was evidence for multiple molecular forms of human red cell esterases from the studies of Micheli and Grabar (1961). They separated hemolysate proteins in an immunoelectrophoretic system, and characterized some of the precipitin arcs thus obtained by specific histochemical staining procedures.

In 1961, Tashian first applied starch gel electrophoresis to the study of human red cell carboxylesterase isoenzymes, and a great deal of the pioneering work was done in his laboratory. Nine bands of esterase activity could be resolved on starch gels by electrophoresis at pH 8.6 using boric acid-NaOH buffers, and α-naphthyl acetate, propionate and butyrate as substrates. The α-naphthol was detected with Fast Blue RR salt. Those esterases that were not inhibited by 10 mM eserine, nor by 1 mM DFP, were designated "A", while those that were inhibited by the DFP but not by the eserine were designated "B", keeping the terminology that had been applied to the serum esterases (see in section 31.2.1). Altogether, eight A-esterase bands could be distinguished on the gel, along with one B-esterase band. One pair of altered A-esterase bands was found in a set of adult identical twins, and the pattern was shared by their mother. This genetic variation was studied further by Tashian and Shaw

Table 31.2 Classification of Carboxylesterases

Esterase Type	Synonyms	Substrates	Inhibitors	Activators
A esterases				
general	aromatic esterases, arylesterases	Ar-OAc > Ar-OBu not aliphatic or choline esters	pCMB, pHMB EDTA, La^{3+}	Ca^{2+}
red cell	acetylesterases	α-NOAc > β-NOAc > αNOPr > αN-OBu	pCMB, IA	
B esterases				
general	simple esterases, alliesterases	Al-esters > Ar-esters	organophosphates	
red cell	butyrylesterases	α-NOBu > β-NOAc > α-NOPr > α-NOAc	DFP	pCMB, IA
C esterases				
red cell	acetylesterases	α-NOAc > β-NOAc > α-NOPr > α-NOBu		pCMB, IA
D esterases	–	MU-OAc > MU-OBu	Hg^{2+}	
Cholinesterases				
red cell	acetylcholinesterase, AChE	choline-OAc > choline-OBu	organophosphates, carbamates, eserine	
plasma	nonspecific cholinesterases, pseudocholinesterases	choline-esters > Al-esters > Ar-esters	organophosphates, eserine	
Carbonic Anhydrases	–	CA_1 MU-OAc > β-NOAc > α-NOAc = MU-OPr CA_2 Fl-diOAc > β-NOAc > α-NOAc = MU-OAc = Fl-OPr	acetazolamide	

Abbreviations: Ar-OAc = aromatic acetate esters; Ar-OBu = aromatic butyrate esters; α-NOAc and β-NOAc = α- and β-naphthyl acetates; α-NOPr = α-naphthyl propionate; α-NOBu = α-naphthyl butyrate; Al = aliphatic; Ar = aromatic; MU-OAc, MU-OPr and MU-OBu = 4-methylumbelliferyl acetate, propionate and butyrate; Fl-diOAc = fluorescein diacetate; Fl-OPr = fluorescein propionate; pCMB = p-chloromercuribenzoate; pHMB = p-hydroxymercuribenzoate; EDTA = ethylene diamine tetraacetic acid; IA = iodoacetamide; DFP = diisopropyl fluorophosphate

(1962). The variant esterase was segregating in three generations of this family, and pedigree data were consistent with its inheritance as an autosomal allele. Somewhat better resolution of the esterase bands was achieved by Tashian and Shaw (1962), and the isoenzymes were characterized further. Accordingly, the classification scheme was changed. Old A1 and A2 were designated "C", A_3–A_6 were re-designated A_1a–A_1d, A_7 was resolved into four bands called A_2a–A_2d, B remained B, and A_8 was identified as carbonic anhydrase I (CA I). In 1965, Tashian described further studies on the separation and characterization of the esterase isozymes, as well as several genetic variants of them. The classification was again changed to reflect the newer findings. The A_1a and A_1b bands were designated "A_1", while the old A_1c and A_1d bands were now called "A_2". The old A_2 region now became "A_3". The most recent scheme is found in the Tashian (1969) review, and is presented in Fig. 31.5. It should be mentioned that Shaw *et al.* (1962) first characterized the carbonic anhydrases (CA I and CA II), which have esterase activity, as "esterases Da_1 and Db". It was very soon clear, however, that these enzymes were carbonic anhydrases (section 32), and they have no relationship to what we now call "esterase D" (see in section 31.3.4).

31.3.3 Genetic variation of the red cell A-esterases

Only a few rare variants of the A-esterases have been reported. The locus controlling the expression of these enzymes in red cells should not, therefore, be regarded as polymorphic. The variant pattern described by Tashian (1961) and Tashian and Shaw (1962) occurred in a White family, and was later called an "AB" phenotype (Tashian, 1965 and 1969). In these terms, an "A" phenotype is the normal pattern, and the variant pattern was thought to represent heterozygosity. A further variant pattern has been identified in a Black person (Tashian, 1965) and was called "AC". An additional example of "AB" was found in a Black individual as well. These variants were identified in a survey of 2638 Caucasian, 623 Black, 366 American Indian and 490 Micronesian bloods. Tashian (1969) has reviewed this material.

31.3.4 Esterase D and its polymorphism

In 1973, Hopkinson *et al.* described a new esterase in human red cells, which had been detected using fluorogenic substrates, 4-methylumbelliferyl acetate and 4-methylumbelliferyl butyrate (MUA and MUB). They called this enzyme Esterase D, and the locus controlling its expression exhibited genetic polymorphism in all populations studied. Esterase D (ESD) could be resolved by starch gel electrophoresis in a number of different buffer systems, but the most satisfactory was found to be Tris-citrate-borate-LiOH, pH 7.2. Electrophoresis was generally carried out at 1.5V/cm for 17 hrs at room temperature. ESD is not detected using α-naphthyl acetate as substrate, but MUA detects A_1 and B esterases in addition to ESD (see in section 31.5). MUB detects ESD and B esterases, while α-naphthyl butyrate detects the B esterase but not ESD.

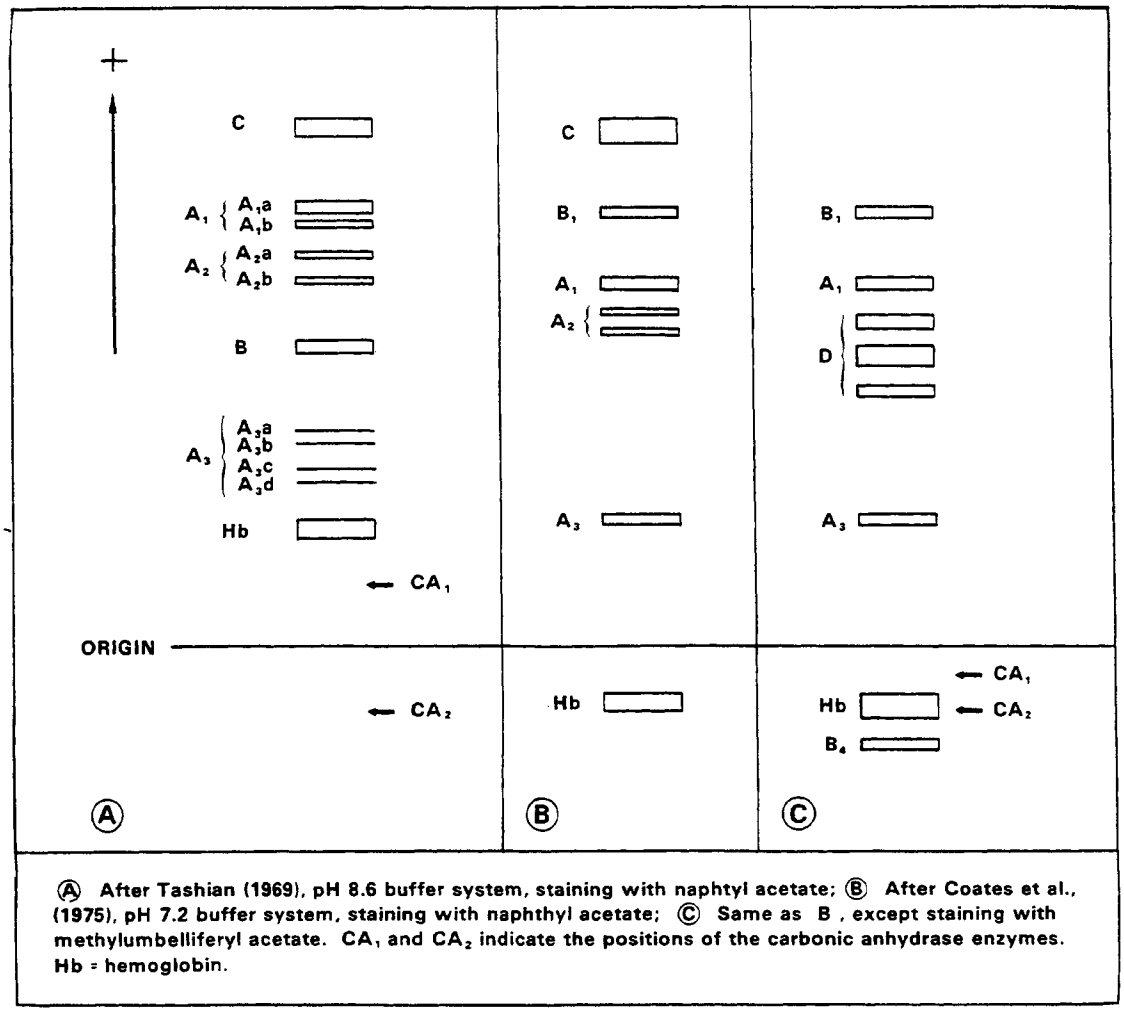
Three principal patterns of ESD were seen in red cell lysates, and were designated ESD 1, 2-1 and 2. The patterns are shown diagramatially in Fig. 31.6. Family studies indicated that these phenotypes represented the expression of a pair of codominant alleles, ESD^1 and ESD^2, at an autosomal locus.The ESD polymorphism was investigated in Europeans, Blacks and Asiatic Indians. ESD^1 was found to be about 0.9 in White and Black people, and about 0.77 in the Indian people. Thus, in White and Black populations, about 80% of people are ESD 1, about 20% are 2-1 and ESD 2 is relatively rare (< 1%). In the Asiatic Indians, there were 63% ESD 1, 28% ESD 2-1 and 9% ESD 2 people. A number of workers have confirmed the single, autosomal allelic pair hypothesis of inheritance for ESD (Benkmann and Goedde, 1974; Ishimoto *et al.*, 1974; Kühnl *et al.*, 1974a; Bender and Frank, 1974). ESD phenotypes have been determined in a number of other populations as well (Welch and Lee, 1974; Kühnl *et al.*, 1974a; Ishimoto *et al.*, 1974; Bender and Frank, 1974; Sensabaugh and Golden, 1976b). A rare allele at the ESD locus, which was called ESD^3, was reported by Bender and Frank (1974). It was detected as an ESD 3-1 (see in Fig. 31.6). An additional allele, ESD^4, was detected by Berg *et al.* (1976) in two members of a family. One was an ESD 4-1, and the other an ESD 4-2. The ESD^4 had been inherited by a daughter from her father. These workers obtained a specimen of the ESD 3-1 blood from Drs. Bender and Frank, and ran all the phenotypes side by side using the original PGM buffer system of Spencer *et al.* (1964b). The patterns are indicated in Fig. 31.6. Grüner and Simeoni (1978) found an ESD 4-1 father with an ESD 4-2 son in a German population. Eriksen and Dissing (1979) reported an apparently new variant which was similar to, but not quite the same as a 3-1. It was not named (or numbered). Sparkes *et al.* (1979) have documented a probable silent allele of ESD, ESD^o, in a family in this country. Patscheider and Dirnhofer (1979) described an extraordinary child who appeared to be heterozygous for an ESD^o allele, and for a silent allele of Gc (section 41.2) as well. Her mother was thought to be ESD 2-0, Gc 2-0, and she had inherited the ESD^o from her mother and the Gc^o from her father.

The *ESD* locus has been tentatively assigned to chromosome 13 (Van Heyningen *et al.*, 1975).

31.3.5 Biochemical studies on esterases

The best studied esterase preparations are not from red cells, nor even from human sources. Krisch (1971) reviewed the biochemical studies on the most highly purified carboxylesterases. There are very few studies on the esterases from human tissues.

It may be noted that Hopkinson *et al.* (1973) characterized ESD and some of the other red cell esterases with respect to substrate specificity using sixteen different esters. ESD was most active with MUA, and hydrolyzed the butyrate and heptanoate esters relatively well. ESD was poorly active with the methylumbelliferyl esters of longer chain fatty acids, and it did not hydrolyze naphthyl esters,



Ⓐ After Tashian (1969), pH 8.6 buffer system, staining with naphtyl acetate; Ⓑ After Coates et al., (1975), pH 7.2 buffer system, staining with naphthyl acetate; Ⓒ Same as B , except staining with methylumbelliferyl acetate. CA_1 and CA_2 indicate the positions of the carbonic anhydrase enzymes. Hb = hemoglobin.

Figure 31.5 Relative Electrophoretic Mobilities of the Red Cell Carboxylesterases

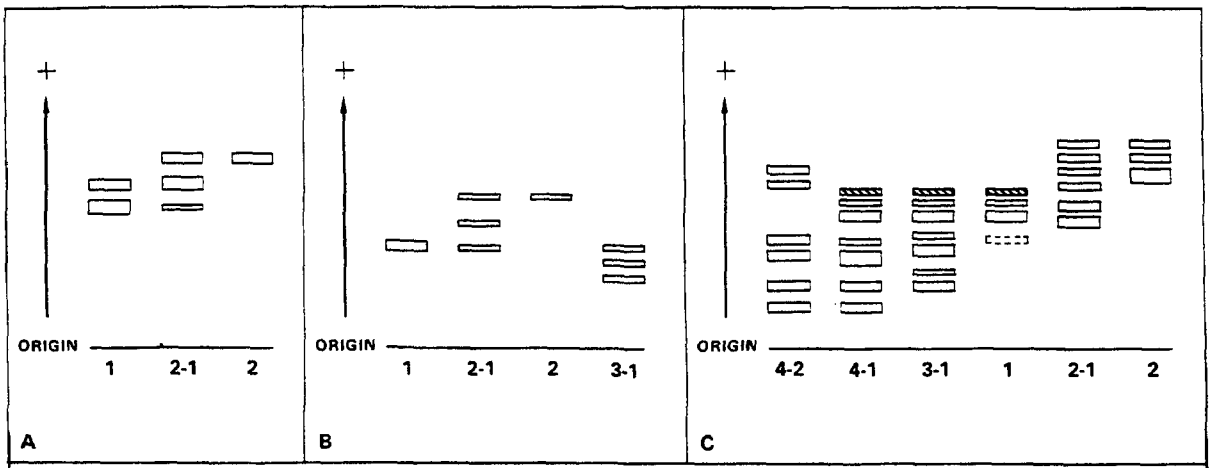
indoxyl acetate, 5-bromoindoxyl acetate, or thiocholine esters. ESD was optimally active at pH 5.0–5.5 with methylumbelliferyl esters, in contrast to the A, B and C esterases, which had pH optima of 7.5–8.0. Stored bloods or hemolysates could sometimes develop a "storage band". This band appeared less frequently in the presence of 20 mM mercaptoethanol. Finally, they noted that the electrophoretic patterns of the phenotypes suggested a dimeric structure for the enzyme, and that the MW of ESD as estimated by gel filtration was about 60,000.

Scott and Wright (1978) carried out a study on the substrate specificity of partially purified isozymes of ESD, and their results showed that methylumbelliferyl esters were the best substrates.

31.3.6 Tissue esterases—Carboxyl esterase classification and a genetic interpretation

Carboxyl esterases occur in a variety of tissues. Most esterases are apparently not very specific about their substrate preferences, and it is primarily this property which has made them difficult to classify. It turns out, too, that there are quite a large number of esterase isozymes in various tissues, and this fact adds to the difficulty.

In 1975, Coates *et al.* conducted an extensive study of tissue carboxylesterases, and tried to formulate a genetic interpretation of them based upon the results. The paper is quite involved, and not easily summarized. Many tissues were examined for esterase activity by electrophoresis, and with a variety of substrates and inhibitors. At least 13 different sets of human esterases, in addition to carbonic anhydrase and cholinesterase, could be identified, and the data indicated that there are at least 9 separate structural gene loci involved in the expression of these isoenzymes. On the basis of the data, Coates *et al.* (1975) suggested a modified nomenclature system for these esterases, in which the historical designations were retained where possible, and in which an effort was made to organize all the available information. The designation "ES" was used for "esterase", and the "A", "B", "C", etc., designation of the types of esterases was retained. Subscripts are used to distinguish sets



A: After Hopkinson et al. (1973), pH 7.2 buffer system. B: After Bender and Frank (1974), pH 7.4 buffer system, showing the 3-1 pattern. C: After Berg et al., (1976), showing the patterns of all known phenotypes, run in PGM, pH 7.4, buffer system. The mobilities of the isozymes with respect to the origin in A, B and C are not comparable, because these representations were taken from different sources.

Figure 31.6 Electrophoretic Patterns of ESD Phenotypes

of isozymes, e.g. ESA_1, ESA_2, etc. The information is summarized in Table 31.3.

There have been a number of reports on esterases in seminal plasma and spermatozoa, but Coates *et al.* (1975) did not examine seminal plasma or sperm cells. As a result, the relationship of the seminal and sperm esterases to the other tissue esterases is not clear (except for ESD). Sperm and seminal esterases were discussed in section 10.9. Blake and Sensabaugh (1976) found that ESD is expressed in sperm cells primarily, and only weakly in seminal plasma. The *ESA*, locus has been shown to be linked to LDH-A, thus assigning it to chromosome 11 (Shows, 1972).

31.4 Medicolegal Application of the Esterases

31.4.1 Disputed parentage

No references were found on the application of the E_1 or the E_2 locus polymorphisms in resolving disputed parentage cases. Chakraborty *et al.* (1974) said that about 1 or 2% of White, and about 0.5% of Black falsely accused fathers would be excluded by E_1 locus phenotyping, according to their calculations.

ESD is employed in a number of laboratories. Prokop and Göhler (1976) noted that 9–10% of falsely accused fathers would be excluded by ESD alone. The value for the British population is 9% as well (Boorman *et al.*, 1977). Polesky *et al.* (1976) said that 8.06% was the figure for this country, and it applied equally to White or Black populations. They recommended the system for parentage cases (Dykes and Polesky, 1977), and said that five exclusions had been obtained in 206 of their cases on the basis of the ESD system alone. The ESD isozyme patterns are fully developed in fetal blood (Chen *et al.*, 1977). The rare silent allele of ESD should give rise to caution in interpreting second order exclusions.

31.4.2 Plasma cholinesterase phenotyping in bloodstains

In 1962, Lehmann and Davies reported that the E_1 locus variants could be determined in bloodstains using 5×10^{-5}M benzoylcholine as substrate and 10^{-5}M dibucaine. They said that bloodstains on cloth and filter paper could be typed. Results were obtained on stains up to 6½ years old, but confirmed for accuracy with the serum of the donor only in stains up to 9 months old. The only other coverage of this subject that was found appeared in Culliford (1971). A procedure was given for the determination of the DN in 1:100 dilute serum, and in bloodstain extracts adjusted to be equivalent to about 1:50 dilute whole blood. Development of this procedure was attributed by Culliford (1971) to Parkin (M.Sc. Dissertation, 1968). Two rapid screening procedures were described as well. The difficulty with the screening procedures (section 31.2.3.7) in bloodstain extracts or hemolyzed specimens is that hemoglobin is present in these samples, and it interferes with the reading of the results because the reaction products are a reddish-brown color. Hemoglobin can be removed from the sample by treatment with CM-Sephadex in a phosphate buffer, pH 6.5, for about 30 min. After centrifugation, the supernatant fluid can be used in the agar gel screening test, essentially the same as the one described by Harris and Robson (1963a).

Another screening procedure relied upon the electrophoretic removal of Hb from the well of the agar gel plate. This procedure was said to have been devised by Wraxall. Here, "inhibited" and "control" plates of agar gel were prepared in Tris-HCl, pH 7, buffer, using RO2-0683 as inhibitor. Wells were punched in the gels, and appropriately diluted test material added. Electrophoresis for about 45 min at about 6.5 V/cm sufficed to allow the Hb to migrate into the gel, and away from the esterase reaction area. These plates were then developed for ChE activity as usual. All samples

Table 31.3 Nomenclature and Properties of Various Tissue Esterases (after Coates et al., 1975)

Esterase Isoenzyme	Genetic Locus	Optimal Substrate(s)	Occurrence in Tissues	Inhibitors
A_1, A_2, A_3	*ESA*,	MU-OAc, α-N-OAc, β-N-OAc; α-N-OPr, ϕ-SAc, Ph-OAc	Pl, Leu, Er	Hg^+
A_4	*ESA*$_4$	α-N-OAc, β-N-OAc	All except Er	–
A_5	*ESA*$_5$	α-N-OAc	All except Er	Hg^+
A_6	*ESA*$_6$	MU-OAc, α-N-OAc	All except Er and Leu	Hg^+
A_7	*ESA*$_7$	–	Foetal Br	–
B_1	*ESB*,	MU-OBu, α-N-OBu, β-N-OBu	All	Hg^+
B_2	*ESB*$_2$	MU-OBu, Fl-OBu, α-N-OBu, α-N-OVa β-N-OBu	All except Er, Leu, Ly, Fi, Re and Ch	E-600, NaF
B_3			All except Er	
B_4	*ESB*$_4$	MU-OBu, Fl-OBu	All	Hg^+
C	*ESC*	α-N-OAc, β-N-OAc	Only Er	E-600, eserine
D	*ESD*	MU-OAc, MU-OPr	All	Hg^+
CA_1	*CA*$_1$	MU-OAc, β-N-OAc, Ph-OAc	Er, Li, Ki, Re, Ch	acetozolamide
CA_2	*CA*$_2$	Fl-diOAc, β-N-OAc	Er, Li, Ki, Re, Ch	acetozolamide

Abbreviations: **Substrates:** MU-OAc, MU-OPr, MU-OBu = 4-methylumbelliferyl acetate, propionate, butyrate; α-N-OAc, α-N-OPr, α-N-OBu, α-N-OVa = α-naphthyl acetate, propionate, butyrate, valerate; β-N-OAc, β-N-OBu = β-naphthyl acetate, butyrate; ϕ-SAc = phenylthioacetate; Ph-OAc = phenolphthalein acetate; Fl-diOAc = fluorescein diacetate; Fl-OBu = fluorescein butyrate

Tissues: Pl - placenta; Leu - leucocytes; Er - erythrocytes; Br - brain; Ly - lymphoid cell lines; Fi - fibroblasts; Re - retina; Ch - choroid; Li - liver; Ki - kidney

giving indications of "intermediate" or "atypical" behavior in either screening test were then subjected to DN determination by the standard method.

Culliford (1971) said that considerable ChE activity is lost when blood dries, but that satisfactory results could be obtained with stains up to 3 months old. After that, activity tended to be so low that differentiation of the inhibition variants was no longer possible. There were no reports found in the literature having to do with the detection of fluoride-inhibition variants in dried blood. The latest material from the London laboratory (MPFSL, 1978) does not mention plasma ChE phenotyping, and this procedure may have been eliminated from routine casework.

The typing of the C_5^+ variant (E_2 locus variant) in bloodstains was also described by Culliford (1971). The best procedure was found to be one devised by Parkin (M.Sc. Dissertation, 1968). Horizontal polyacrylamide gels with a starch insert were employed (see description in section 2.3.7.2), using Tris-succinate, pH 4.8, gel buffers and citric acid-NaOH, pH 4.8, bridge buffer. Threads from bloodstains were inserted directly into the gel after treatment with 1% mercaptoethanol. The mercaptoethanol was necessary to remove the "storage bands", which when present made the diagnosis of C_5^+ difficult. Examination of Figure 3–43 in Culliford (1971) shows this effect. The C_5^+ variant could be typed in bloodstains for a matter of months. The latest material from this laboratory, however, does not mention C_5^+ typing in bloodstains (MPFSL, 1978). Joshi *et al.* (1979) carried out studies on the typability of C_5^+ in bloodstains kept under a number of different conditions, stains from postmortem bloods and case bloodstains. Stains in hot, humid conditions lasted only about a day, but stains at room temperature at lower humidity could be typed for 12 days. The enzyme could be detected for a much longer time in stains kept at 4° or frozen. Stains from postmortem bloods retained activity for at least a month at room temperature. About 50% of the case stains were typable at 20 days age, but less than 2% of them at 40 days age or older.

31.4.3 ESD phenotyping in bloodstains

ESD phenotyping in bloodstains was first described by Blake and Sensabaugh in 1974, and much of this work appeared in 1975 in a separate publication. At least two buffer systems were found to be suitable for ESD typing. One was the original ACP buffer system of Hopkinson *et al.* (1963) (section 29.2), and the other was the original PGM buffer system of Spencer *et al.* (1964b) (section 27.2.1). The PGM buffer system was regarded as the preferable one. It was noted that, because ESD phenotypes are detected by fluorescence of methylumbelliferone under UV light at the sites of activity, that a gel run for ESD could be rinsed off and stained for PGM. It was also pointed out that the methylumbelliferyl esters are exceedingly unstable in basic solution, and that spontaneous hydrolysis of them is favored by higher Tris concentration and by imidazole, even at pH 7. As a result, phosphate or acetate buffers at pH 5.3 were recommended for the reaction buffer.

Parkin and Adams (1975) described a procedure for ESD typing in bloodstains, using a boric acid-LiOH bridge buffer and a Tris-citrate-boric acid-LiOH gel buffer, at pH 7.2. Hemolysates were diluted 1:1 with 50 mM DTT before electrophoresis, and bloodstained threads were inserted directly into the gel after soaking in 50 mM DTT. Stains up to 3 weeks old could be typed. Hayward and Bosworth (1975) described another procedure. They employed citrate-phosphate buffers at pH 5.9 for electrophoresis, and they recommended the substitution of MUB for MUA as substrate. The patterns obtained were less bright, they said, but other esterases were not stained, thus simplifying interpretation.

Grunbaum *et al.* (1978a) described a procedure for ESD phenotyping on cellulose acetate membranes which was applicable to hemolysates and to bloodstains up to 2 months old.

Jay and Philp (1979) found that bloodstains of type 1 or 2-1 could be typed for up to 4 weeks. Whole blood was typable for a few days if kept at 37°, but for 6 weeks if kept at 3°. As has been noted above, Blake and Sensabaugh (1974) recommended the simultaneous phenotyping of ESD and PGM in the same gel. Wraxall and Stolorow (1978) recently described a procedure, using the original PGM buffer system, for the simultaneous typing of ESD, PGM and GLO.

31.4.4 ESD phenotyping in other tissues

ESD occurred in significant concentrations in all tissues tested by Coates *et al.* (1975) (see in Table 31.3). Under favorable conditions, ESD phenotypes could be expected to be determinable in any of these tissues, which included muscle, kidney, heart, liver and many others. The enzyme was found in testicular tissue but seminal plasma and spermatozoa were not tested. Blake (1976) and Blake and Sensabaugh (1974 and 1976) have shown that ESD occurs primarily in spermatozoa, and is found in only trace amounts in seminal plasma. Oepen *et al.* (1980) said that they could not detect ESD in sperm, even in lysates. Blake and Sensabaugh (1978) have estimated, however, that ESD should be determinable in 10 $\mu\ell$ or more of whole semen. The estimate was based on a sperm count of 8×10^7 spermatozoa per mℓ semen. Yoshida *et al.* (1979) showed that ESD can be typed in hair roots.

31.5 The Distribution of Cholinesterase and ESD Phenotypes in U.S. Populations

The data are shown in Table 31.4. Cholinesterase polymorphisms have not been widely applied to bloodstain individualization. Neither of them is very powerful in making distinctions in the population. The discrimination index would be of the order of 0.05 for Caucasians and Hispanics with E_1 locus variants, and lower for Negroes and Orientals. For the E_2 locus, only about 5% of Caucasians are C_5^+, and even fewer Blacks. The DI for the ESD polymorphism is of the order of 0.35 for Whites, 0.29 for Blacks, 0.42 for Hispanics and 0.6 for Orientals. Steegmüller (1975) reviewed world population distributions for cholinesterase polymorphisms, and gave an analysis of the data.

Table 31.4 Distribution of Cholinesterase and ESD Phenotypes in U.S. Populations

Population	Total	E_1 Phenotype — Number (Percent) U	I	A	E_2 Phenotype — Number (Percent) C_s^+	C_s^-	ESD Phenotype — Number (Percent) 1	2-1	2	*ESD'* ★	Reference
CAUCASIAN											
Seattle, WA	246	238 (96.7)	8 (3.3)	0							Motulsky and Morrow, 1968
U.S. Naval Personnel in Japan	137				14 (5.25)	123 (94.75)					Omoto and Harada, 1968 [11]
National Sample [3]	1,494	1,446 (96.8)	49 (3.3)	2 (0.1)							Lubin et al., 1971
Mississippi [3]	142	134 (94.4)	8 (5.6)	0							Lubin et al., 1971
Orange County, CA [3]	181						130 (72)	44 (24)	7 (4)	0.840	Fitzpatrick et al., 1976
Minnesota	506						422 (83.4)	78 (15.4)	6 (1.2)	0.911	Dykes and Polesky, 1977
Pittsburgh/Allegheny County, PA	545						(78.7)	(19.8)	(1.5)	–	Mortimer et al., 1978
California	5,377						(79.5)	(19.3)	(1.2)	0.892	Grunbaum et al., 1978b
Detroit, MI	503						392 (77.9)	106 (21.1)	5 (1.0)	0.885	Stolorow et al., 1979 [10]
Miami/Dade County, FL	367						286 (77.9)	76 (20.7)	5 (1.4)	0.883	Stuver, 1979 [10]
Los Angeles, CA	335						252 (75.2)	76 (22.7)	7 (2.1)	0.866	Siglar, 1979 [10]
NEGRO											
Seattle, WA	100				2 (2)	98 (98)					Robson and Harris, 1966
Seattle, WA	317				16 (2.6)	301 (97.4)					Ashton and Simpson, 1966
Seattle, WA	115	112 (97.4)	3 (2.6)	0							Whittaker, 1968d
Seattle, WA	666	659 (98.9)	7 (1.1)	0							Motulsky and Morrow, 1968
National Sample [3]	347	346 (99.7)	1 (0.3)	0							Lubin et al., 1971
Mississippi [3]	118	118 (100)	0	0							Lubin et al., 1971
Pittsburgh/Allegheny County, PA	152						(76.3)	(23.7)	(0)	–	Mortimer et al., 1978

Table 31.4 (Cont'd.)

Population	Total	E_1 Phenotype — Number (Percent) U	I	A	E_2 Phenotype — Number (Percent) C_2^+	C_2^-	ESD Phenotype — Number (Percent) 1	2-1	2	*ESD'*★	Reference
California	973						(83.6)	(16.0)	(0.4)	0.916	Grunbaum et al., 1978b
Detroit, MI	504						424 (84.1)	76 (15.1)	4 (0.8)	0.917	Stolorow et al., 1979 [16]
Miami/Dade County, FL	343						286 (83.4)	54 (15.7)	3 (0.9)	0.913	Stuver, 1979 [18]
Los Angeles, CA	146						121 (82.9)	23 (15.8)	2 (1.4)	0.908	Siglar, 1979 [19]
HISPANIC											
San Ysidro, CA [4]	105	99 (94.3)	6 (5.7)	0							Lubin et al., 1971
California [5]	1,580						(73.9)	(23.8)	(2.3)	0.858	Grunbaum et al., 1978b
Miami/Dade County, FL	360						259 (71.9)	89 (24.7)	12 (3.3)	0.843	Stuver, 1979 [18]
Los Angeles, CA [6]	156						118 (75.6)	35 (22.4)	3 (1.9)	0.869	Siglar, 1979 [19]
ORIENTAL											
Seattle, WA [7]	426	422 (99.1)	4 (0.9)	0							Motulsky and Morrow, 1968
Seattle, WA [8]	55	73 (100)	0	0							Whittaker, 1968d
San Francisco, CA [8]	111						17 (30.9)	30 (54.5)	8 (14.5)	0.582	Sensabaugh and Golden, 1976b
San Francisco, CA [9]	73						40 (36.0)	56 (50.5)	15 (13.5)	0.612	Sensabaugh and Golden, 1976b
ASIAN											
California and Hawaii	3,029						(41.6)	(44.2)	(14.2)	0.637	Grunbaum et al., 1978b

[1] Cited by Mourant et al., 1976

[2] Random sample of unrelated children

[3] About 15% were Mexican-American

[4] Mexican

[5] "Chicano/Amerindian"

[6] Primarily Mexican

[7] Primarily Japanese

[8] Japanese

[9] Chinese

[10] And see Shaler, 1978

★ Gene frequency

SECTION 32. CARBONIC ANHYDRASE

32.1 Recognition of Carbonic Anhydrase

Carbonic anhydrase (CA; carbonate dehydratase; carbonate hydro-lyase; E.C. 4.2.1.1) catalyzes the reversible conversion of carbonic acid to CO_2 and H_2O. Its physiological importance is based on the fact that much of the CO_2 produced in tissues as a product of cellular respiration is transported in blood as H_2CO_3 (or, more correctly, as $H^+ + HCO_3^-$). The enzyme plays an essential role in the conversion of dissolved carbonate to CO_2 in the lungs. The early physiologists thought that hemoglobin was primarily involved in CO_2 transport, and they were partly right. Some CO_2 is bound to Hb, but not the majority. Studies on the $H_2CO_3 \rightleftarrows H_2O + CO_2$ equilibrium and its rate of reaction indicated that the spontaneous rate was too slow to meet the needs of physiological function, and that hemolyzed blood significantly increased the conversion of bicarbonate to CO_2 (Henriques, 1928). He thought that Hb was catalyzing the reaction. In 1932, Meldrum and Roughton showed that the catalytic activity was distinct from hemoglobin and from its components, and proposed to call it "carbonic anhydrase" (Meldrum and Roughton, 1932a, 1932b and 1932c). The enzyme is found primarily in red cells, and Meldrum and Roughton (1933) purified it and described many of its properties. Stadie and O'Brien (1933a and 1933b) confirmed these findings, apparently independently. Keilen and Mann (1939 and 1940) established that CA was a Zn-enzyme, and this finding established a physiological role for zinc for the first time. The earlier studies on CA were reviewed by Roughton in 1935, and again in 1943.

32.2 Multiple Forms of Carbonic Anhydrase

32.2.1 Recognition of CA isoenzymes

In 1960, it began to be shown that human CA occurs in different molecular forms. At least four different laboratories, working independently and using somewhat different methods, were involved. Most of the purification work has been carried out on the human and the bovine red cell enzymes, and these have been extensively characterized. In 1960, Linskog found that two distinct forms of CA were isolated in the purification of the bovine red cell enzyme. This was soon found to be the case for the human enzyme by the Uppsala group (Nyman, 1961). Refinement of the purification procedures led to the finding of a third form of CA (Nyman and Lindskog, 1964). Around the same time, similar results were obtained in Derrien's laboratory in Marseilles (Laurent *et al.*, 1965 and 1966; Reynaud *et al.*, 1965), and in Edsall's laboratory at Harvard (Rickli and Edsall, 1962; Rickli *et al.*, 1964). Everyone used a somewhat different nomenclature for a time, but in 1964, all the laboratories had agreed to call the three components carbonic anhydrases A, B and C (Rickli *et al.*, 1964). CA-A was the minor, low specific activity, component, CA-B was the major low specific activity component, and CA-C was the high specific activity one. In 1962, Shaw *et al.* in Tashian's laboratory found two forms of CA in human red cells, which could be separated by starch gel electrophoresis at pH 8.7 in borate buffers. These forms were detected by their esterase activity, and were first called esterases "Da" and "Db", but it was strongly suspected, and soon proven (Tashian *et al.*, 1963) that these were carbonic anhydrases (see in section 31.3.2). On the basis of similarities and differences in the properties of the different forms and of their genetic control, Tashian *et al.* (1963) designated the two major CA's as CA I and CA II, and these correspond to the CA-B and CA-C, respectively, of Rickli *et al.* (1964).

In addition to the two major isozymes, different numbers of isozymes which occur in very much smaller amounts have been observed by different investigators. These forms may be seen as minor electrophoretic bands, or they can be obtained as separate fractions in purification sequences. Their nature is not completely clear, but there is evidence which strongly suggests that they are not the products of different genetic loci from those which control CA-I (CA-B) and CA II (CA-C). The minor component CA-A in the "A", "B", "C" nomenclature of Rickli *et al.* (1964) is designated CA I (+1) by Tashian (Tashian, 1969). This designation is based upon a systematic scheme for designating minor forms based upon electrophoretic mobilities (Tashian, 1969). The scheme is shown in Figure 32.1. CA-A is known to be identical in its properties to CA-B (Laurent *et al.*, 1966). Tashian (1969) suggested that these minor forms may be conformational isomers of the major CA isozymes, by analogy to the very similar behavior of chicken mitochondrial malate dehydrogenase observed by Kitto *et al.* (1966), who referred to the different forms as "conformers". Funakoshi and Deutsch (1969) have examined the multiple isozymic forms of CA in some detail. They found that all the forms are similar to one of the major ones, and that many of the "minor" forms can be generated *in vitro* by incubation of the major forms at relatively high pH. They use capital letter nomenclature (major isozymes are CA-B and CA-C), and they designated the minor forms with a series of letters as well. The correlation between these forms and those represented in Figure 32.1 is not clear. Funakoshi and Deutsch (1969) said that these forms may differ from one another in the number of amide groups, i.e., one or more of the Gln or Asn residues of the native molecule may be hydrolyzed to Glu or Asp.

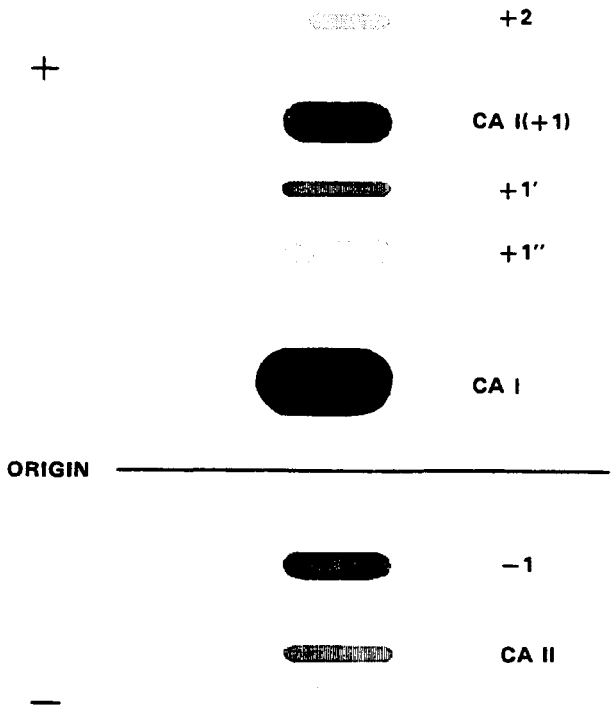


Figure 32.1 Designations of Minor Isozymes in Relation to the Major Isozymes of CA (after Tashian, 1969)

32.2.2 Genetics and nomenclature of CA isoenzymes

Based primarily upon the genetic variation that has been found in the carbonic anhydrases (section 32.3), the CA I (CA-B) and CA II (CA-C) isozymes are believed to be controlled by two different genetic loci. All the evidence supports this view. These loci will be designated CA_I and CA_{II}, after Hopkinson *et al.* (1974). The nomenclature issue is apparently still not settled (Tashian and Carter, 1976). The biochemists still tend to use the "A", "B", "C" nomenclature, while investigators with a genetic orientation have mostly adopted Tashian's nomenclature. The use of the symbols CA_I and CA_{II} to represent the genetic loci, and the designation of the different alleles, especially of the polymorphic CA_{II} locus, by superscript numbers is consistent with general nomenclature trends in human biochemical genetics. CA nomenclature is due for a revision, based upon some general agreement among all workers, and this may come when the sequences of the CA_I locus variants have been worked out.

32.3 Genetic Variation of CA

32.3.1 Genetic variation at the CA_I locus

The CA_I locus cannot be regarded as polymorphic in most populations. Most of the variants that have been found are exceedingly rare. Many of them have been thoroughly studied, and the results have given much information about the genetic control of CA's, but because none of them occur at polymorphic levels in populations in this country, they are not useful as genetic markers.

The first CA_I locus variant was detected by Shaw *et al.* in 1962 in a Mongoloid (trisomic 21) individual. The father and paternal grandmother of the propositus had the variant as well, and it was unrelated to the Mongolism. This variant was called "Da_2" at first, based on the original designation of CA I as "esterase Da". The name was soon changed, however, to CA Ib, where CA Ia is the designation of the usual kind of CA I (Tashian *et al.*, 1963). These same investigators also described the second variant, called CA Ic, in a group of people called Chamorros in Western Micronesia. There are now something more than 10 different variants known, all representing heterozygosity at CA_I of a variant allele with the common allele. Some of the variant enzymes have been characterized rather extensively, and in some cases the amino acid substitution has been determined. The variants are shown in Table 32.1, and have been well reviewed by Tashian and Carter (1976). An additional variant, CA $I_{Nagasaki\ I'}$ having the amino acid substitution 76 Arg → Gln, has been described by Goriki *et al.* (1979). There are occasional reports of quantitative variation, in which the synthesis of one of the isozymes appeared to be greatly reduced. Rieder and Weatherall (1964), for example, described a young Black girl in whom CA I activity was very low, but CA II was normal.

Table 32.1 CA_I Locus Variants

Variant	Ethnic Background	Where Found	Amino Acid Substitution	Reference(s)
Ib Michigan	Caucasian	USA	–	Show et al., (1962); Tashian (1969)
Ie Michigan	Caucasian	USA	–	Tashian et al. (1968)
P_{Mut}	Caucasian	USA	236 Asp—Val	Funakoshi and Deutsch (1970)
Ie Portsmouth	Caucasian	England	255 Thr—Arg	Carter et al., (1972); Tashian and Carter (1976)
Ie Hull	Caucasian	England	225 Gln—Lys or Arg	Carter et al. (1972); Tashian and Carter (1976)
If London	Caucasian	England	102 Glu—Lys	Carter et al. (1973)
Id Michigan; P	Negro	USA	100 Thr—Lys	Tashian (1965); Shows (1967); Moore et al. (1973); Tashian and Carter (1976)
Ih Jackson	Negro	USA		Tashian and Carter (1976)
Ih Jamaica	Negro	Jamaica		Tashian and Carter (1976)
Ic Guam	Asian	Mariana Islands	253 Gly—Arg	Tashian et al. (1963); Tashian et al. (1966)
Ic★	Asian	Phillipines	–	Lie-Injo (1967)
Ic★	Asian	Indonesia	–	Lie-Injo and Poey-Oey (1970)
Ic★	Asian	Malaysia	–	Lie-Injo et al. (1971)
I "Malaysia"	Asian	Malaysia	–	Lie-Injo et al. (1971)
Ih Hiroshima	Oriental	Japan	Maybe 16 Trp—Arg	Ueda (1974)

★ May be the same as CA Ic Guam

32.3.2 Genetic variation at the CA_{II} locus

In 1971, Moore *et al.* described a variant form of CA-C (CA II), which they called "H". Its electrophoretic mobility was close to that of CA-B (CA I); that is, the variant H isozyme ran anodically, whereas the usual kind of CA II isozyme ran cathodically. In some 222 Black people from various American cities, 18% appeared to be heterozygous for the H isozyme, and about 1% homozygous for it. The phenotypes were designated CC, CH and HH, and family studies which followed (Moore *et al.*, 1973) were consistent with a two allele, codominant, autosomal pattern of inheritance. Subsequent population and family studies have confirmed the postulated mode of inheritance, and the fact that the variant CA II occurs at polymorphic levels in a number of Black populations, including those in this country, in London and in several African nations (Carter, 1972; Moore *et al.*, 1973; Hopkinson *et al.*, 1974; Welch, 1975).

In 1974, Hopkinson *et al.* examined the products of the CA loci using fluorogenic substrates. They made the interesting (and, for phenotyping purposes, extremely useful) discovery that CA_I isozymes react preferentially with methylumbelliferyl acetate, while those of CA_{II} react preferentially with fluorescein diacetate. Thus, an electrophoretic plate stained with MUA reveals only the CA_I isozymes, while one stained with fluorescein diacetate reveals the CA_{II} isozymes. Fluorescein diacetate is, therefore, the preferred substrate for phenotyping CA_{II} isoenzymes (see in Figure 32.2). Hopkinson *et al.* (1974) referred to the "H" phenotype as a CA II 2, and to the "CH" heterozygote as a Ca II 2-1. The alleles determining these phenotypes are CA_{II}^1 and CA_{II}^2, and this usage was adopted by Tashian and Carter (1976). The polymorphism is, thus far, restricted to Black populations. Hughes (1978) described a previously unrecognized phenotype in England, which was called CA_{II} 3-1.

32.4 Biochemical Studies on the CA Enzymes

Since the red cell is a convenient source of CA, many studies have been done on the enzyme from human blood. The major forms of both CA I and CA II have been extensively purified and characterized, as have a number of the variant forms (see in section 32.2.1). For reviews, see Edsall (1968), Linskog *et al.* (1971) and Tashian and Carter (1976). Most purifications have been carried out by subjecting hemoglobin-free hemolysates to gel filtration and/or ion exchange chromatography. CA I and CA II have also been purified by affinity chromatography (Johansen, 1976). The

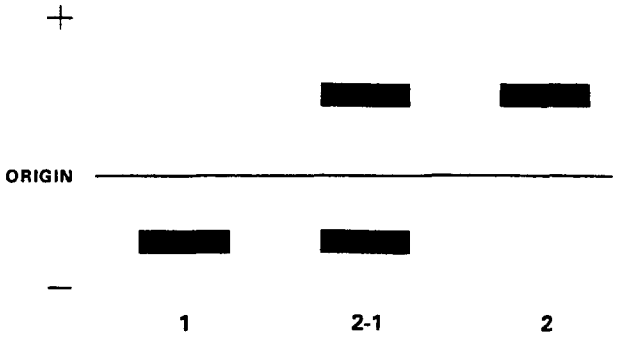


Figure 32.2 Electrophoretic Phenotypes of CA II Isoenzymes Electrophoresis in Gels at pH 8.6, and Staining with Fluorescein Diacetate.

MW's of both enzymes are close to 29,000. Both consist of single polypeptide chains of about 260 amino acid residues, and the sequences are known (see Tashian and Carter, 1976). There is one Zn associated with each molecule, and there are no intramolecular disulfide bridges. The high resolution crystal structures have been determined (see Lindskog *et al.*, 1971). CA will catalyze the hydration of aldehydes, and the hydrolysis of esters in addition to the reversible conversion of CO_2 and H_2O to carbonic acid. The turnover number for the reaction with CO_2 is among the highest known for any enzyme-catalyzed reaction. The Zn^{++} ion is required for catalytic activity, although the binding constant for several other divalent metal ions is higher (Lindskog and Nyman, 1964). CA is inhibited by a number of different things, including most monovalent anions, sulfonamides and some metal ions.

32.5 Red Cell CA Variation in Infrahuman Species—Phylogenetic Relationships

A large number of studies have been conducted on the CA isozymes of primate species other than humans in an effort to better understand the phylogeny of this genetic variation (Tashian, 1965 and 1969; Tashian *et al.*, 1968; Tashian and Carter, 1976). Many primate species have two CA loci, and a number of them show genetic variation at one or both of them. A number of mammals other than primates also have two CA loci, but there are some which appear to have only one. Based upon these studies, and on the sequences of a number of carbonic anhydrases from different species, Tashian has suggested that the two gene loci which occur in some mammals arose by gene duplication during evolution, the CA II probably being the "older" locus.

32.6 Medicolegal Applications

No published papers on the application of CA to disputed parentage were found in the literature that was examined. There is no reason why the CA II polymorphism could not be applied to disputed paternity cases involving Black people, and this application was, in fact, suggested by Moore *et al.* (1973). The gene frequencies of CA_{II}^{1} and CA_{II}^{2} appear to be about 0.9 and 0.1, respectively, in U.S. Black populations. The probability of excluding a falsely accused father with CA II alone would thus be about 8%. The enzymes are fully developed at birth (Moynihan, 1977).

CA_{II} typing in bloodstains has developed quite recently. Hughes (1978) described a procedure on 10% starch gels using a Tris-boric acid-EDTA buffer at pH 8.5 at different strengths for bridge and gel. A very similar procedure is given in MPFSL (1978). CA_{II} phenotypes can be determined on starch-agarose gels, using the multisystem procedure described by Wraxall and Stolorow (1978). This system is designed to allow simultaneous typing of PGM, ESD and GLO. The CA_{II} isozymes can be detected around the origin area of the plate, the CA_{II} staining being carried out along with ESD staining, and prior to PGM and GLO detection. The CA_{II} phenotypes can be observed in bloodstains in this system (Matthews and Stolorow, 1981). These authors have noted, however, that this electrophoretic system should be used to screen samples for CA_{II} phenotypes and not for positive diagnosis. Samples which appear to be types 2-1 or 2 can be confirmed in a more optimal typing system, such as that of Hughes (1978).

32.7 The Distributon of CA_{II} Phenotypes in U.S. Populations

There is very little data on this particular polymorphism. Moore *et al.* (1971) found 180 (81.1%) CA II 1, 39 (17.8%) CA II 2-1, and 3 (1.4%) CA II 2 among 222 Blacks from a number of cities. Tashian and Carter (1976) said they found 103 (80.0%) 1, 23 (18.0%) 2-1 and 2 (1.6%) 2 among 128 U.S. Blacks. Stolorow *et al.* (1979) found 423 (83.9%) 1, 75 (14.9%) 2-1 and 6 (1.2%) 2 types in 504 people in greater Detroit. Mortimer *et al.* (1978) tested 646 people in the greater Pittsburgh area and found the CA_{II}^{1} frequency to be 0.902. The CA_{II}^{1} frequencies in the other studies were 0.914 (Stolorow *et al.*, 1979), 0.899 (Moore *et al.*, 1971) and 0.895 (Tashian and Carter, 1976).

SECTION 33. GLUCOSE–6–PHOSPHATE DEHYDROGENASE AND 6–PHOSPHOGLUCONATE DEHYDROGENASE

33.1 Glucose–6–Phosphate Dehydrogenase (Glc6PD; Gd; Zwischenferment; D-Glc6P:NADP$^+$ 1–oxidoreductase; E.C. 1.1.1.49)

33.1.1 Recognition of Glc6PD

The recognition of Glc6PD, the characterization of its properties, metabolic role, and those of other associated reactions, form a significant part of the history of biochemistry itself in the present century. The review by Noltmann and Kuby (1963) should be read for the details. The enzyme was first described in mammalian red cells by Warburg and Christian in 1931. At the time, the enzyme was regarded as having an "intermediary" function in the transfer of hydrogen from Glc–6P through NADP and "old yellow enzyme" to methylene blue (or oxygen) (Warburg and Christian, 1932), and it was given the name "zwischenferment" (Warburg and Christian, 1933). In 1935, Ogston and Green suggested that the name "zwischenferment" was uninformative as to the enzyme's function, and that it be called "hexose–6–phosphate dehydrogenase". Negelein and Haas (1935) objected to this suggestion, saying: "Das Zwischenferment ist aber so wenig eine Dehydrogenase, wie das Globin des Hämoglobins ein Sauerstoffüberträger ist." [Zwischenferment is as little a dehydrogenase as the globin of hemoglobin is an oxygen carrier]. They thought that Zwischenferment was the apoenzyme and that NADP (then called Coenzyme II) was the prosthetic group responsible for the oxidoreductase activity. There is some truth in this thinking, but the enzyme ultimately came to be known as Glc–6–phosphate dehydrogenase. The first example of Glc–6–PD that was highly purified and characterized came from brewer's yeast.

33.1.2 The Relationship of Glc6PD and Hemolytic Anemia—Recognition of Genetic Variation in Glc6PD

Genetic variation in Glc6PD came to be noticed and extensively studied as the result of clinical observations on the induction of acute episodes of hemolytic anemia in certain individuals by drugs. The first such observation came about when it was noted that the antimalarial drug pamaquine, an 8–aminoquinoline derivative introduced in 1926, caused acute, and even fatal, episodes of hemolytic anemia in certain patients. Dozens of studies failed to reveal the reasons for this reaction, and why only certain people were susceptible to it. A number of observations were made which were not understood at the time, but which seem significant in the light of hindsight. The development of Heinz bodies in the red cells of patients undergoing the drug reaction were noted. Heinz (or Heinz-Ehrlich) bodies are inclusion bodies seen in blood smears resulting from oxidative damage to hemoglobin. The pamaquine sensitivity was observed to be familial in some cases, and it was also noted that there were differences in the number of susceptible individuals in different racial and ethnic groups. These effects could not be sorted out, however, until after 1950, when it became possible to develop *in vitro* tests for the detection of the abnormalities.

In 1950, a therapeutically more effective antimalarial agent, called primaquine, was introduced (Edgcomb *et al.*, 1950). Primaquine, like its predecessor, is an 8–aminoquinoline derivative. In 1954, Dern *et al.* demonstrated that primaquine-induced hemolytic anemia was the result of some kind of defect within the red cells themselves. Biochemical studies of red cells from primaquine-sensitive individuals were carried out, and revealed that their reduced glutathione (GSH) content was uniformly lower than that of normal cells (Beutler *et al.*, 1955). In addition, administration of the drug to sensitive people caused rapid reductions in their red cell GSH levels (Beutler, 1957). Subsequent examination of the pathways of GSH metabolism in red cells disclosed that the primary defect in primaquine-sensitive red cells was a deficiency of Glc–6PD (Carson *et al.*, 1956; Gross *et al.*, 1958; Gross and Marks, 1958). Beutler (1957) found that the compound acetylphenylhydrazine would deplete primaquine-sensitive, but not primaquine-insensitive, red cells of their reduced glutathione *in vitro*, and used this as the basis for a hematological test for primaquine sensitivity. It was called the "glutathione stability test". This material was excellently reviewed in detail by Beutler in 1959.

Primaquine sensitivity tends to be more frequent in certain racial and ethnic groups than in others, and is nearly always associated with Glc6PD deficiency. As studies of this phenomenon progressed, it became clear that there is more than one kind of Glc6PD deficiency, and that the different Glc6PD variants were distinguishable on the basis of the biochemical properties of the enzymes, and in some cases on the resulting clinical manifestations.

Glc6PD deficiency, leading to primaquine sensitivity, was recognized early to occur in appreciable frequencies in Black people of African origin. Most people suffering from the deficiency are clinically asymptomatic unless challenged by certain drugs. Many substances, in addition to the 8-aminoquinoline antimalarials, are now known which will induce hemolytic anemia in susceptible subjects. Depending upon the individual, the clinical course of the hemolytic anemia may be relatively mild or quite severe. Glc6PD deficiency in non-Black populations is similar, in that symptoms

are not usually apparent unless there is exposure to drug stress. There are some hematological differences between the syndromes as manifested in Blacks and Whites, however, and Marks and Gross (1959) suggested that these were reflections of genetic differences.

In certain people, the ingestion of fava beans (*Vicia faba*) induces hemolytic anemia. This syndrome has been called favism, and some earlier workers drew attention to the similarity between it and antimalarial drug sensitivity (see in Beutler, 1966). Favism tends to be particularly characteristic of Mediterraneans, and it is known to be familial. It is also associated with GSH instability and with Glc6PD deficiency (Sansone and Segni 1956 and 1957; Szeinberg *et al.*, 1958a and 1958b). There is something more to it though, since many people with Glc6PD deficiency do not react to fava beans.

There are some cases of what is called congenital nonspherocytic hemolytic anemia (CNHA) associated with Glc6PD deficiency (Newton *et al.*, 1958; McGovern *et al.*, 1958; Zinkham and Lenhard, 1959; Beutler *et al.*, 1968). These cases represent less common forms of Glc6PD deficiency and many of these people suffer from anemia in the absence of drugs. The anemia is often mild, and seen primarily in the neonatal period.

Glucose is the major energy source of the red cell, and the hematological mechanisms underlying red cell enzyme disorders reflect disturbances in glucose metabolism. A simplified diagram of major glucose metabolizing pathways is shown in Figure 33.1. Glc-6PD represents a branchpoint. Glucose can be metabolized via the Embden-Meyerhof pathway (glycolysis) or via the hexose monophosphate pathway (pentose pathway). In normal, unstressed red cells, about 90% of the glucose is metabolized by glycolysis. The Glc-6PD reaction is the principal regulatory step for the hexose monophosphate pathway, the major physiological functions of which are the production of NADPH and the formation of five carbon sugars. Under normal circumstances, the Glc-6P level of the red cell is well below that required for maximal Glc-6PD activity, and so is the level of NADP. Further, NADPH competitively inhibits the enzyme. The rate of metabolism via the hexose monophosphate pathway can thus be readily altered. Perhaps the major function of the hexose monophosphate pathway is the production of sufficient NADPH to keep oxidized glutathione reduced by way of the glutathione reductase reaction (Figure 33.1). Glutathione reductase also reduces the mixed disulfides of glutathione and protein (including hemoglobin). Oxidized glutathione is produced by the glutathione peroxidase reaction which rids the cell of peroxides. These may arise through the action of superoxide dismutase (see section 37.4).

Red cell Glc-6PD deficiencies can thus have far reaching physiological consequences. The consequences in any given instance are complicated, however, by the fact that many of the genes producing deficiency syndromes code for Glc-6PD molecules with altered kinetic properties. This subject has been well reviewed by Beutler (1978).

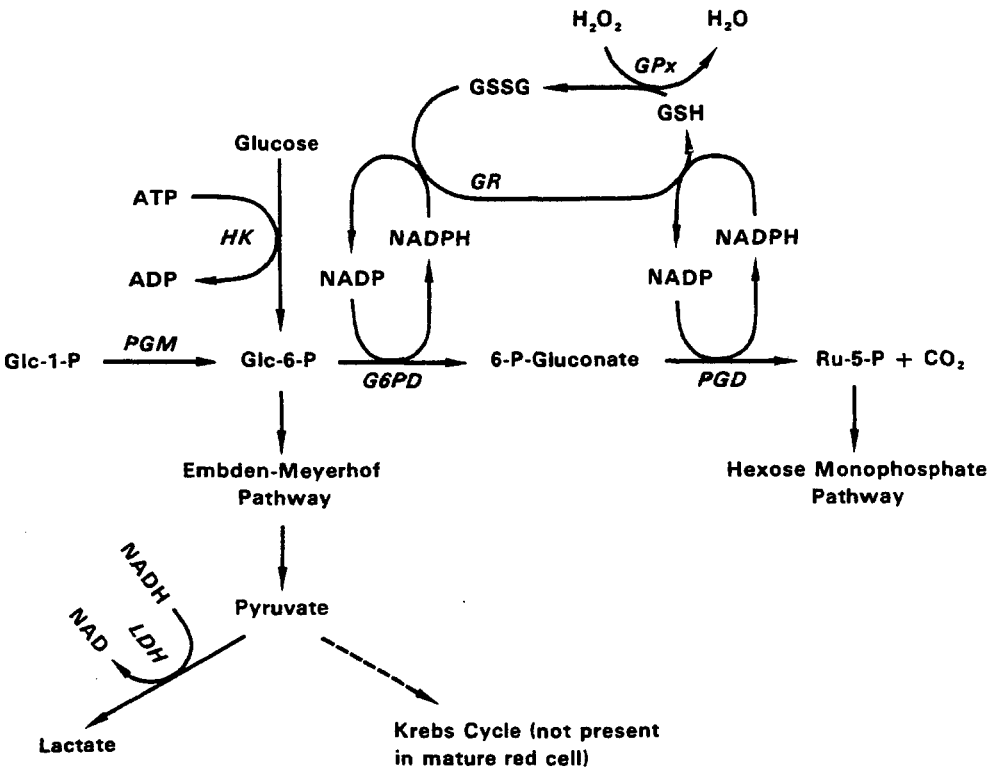
33.1.3 The genetic variants of Glc-6PD

A great deal of work has been carried out on the biochemical genetics of Glc-6PD, and many variant kinds of the enzyme have been described. Some differ from the normal enzyme in electrophoretic mobility, but many do not, and are characterized by differences in kinetic properties, activity or thermostability.

In 1962, Boyer and Porter reported that Glc-6PD in American Blacks showed electrophoretic heterogeneity. Three types could be distinguished, and were called A, B and AB. The sex distribution of the phenotypes was such that the only logical explanation for it was X-linkage of the Glc-6PD locus. Only women were ever heterozygous. Black males with Glc-6PD deficiency were electrophoretically type A (the Glc-6PD type could be determined from leucocytes, which are not deficient even in red cell-deficient subjects). This suggested that there were three alleles, an A-normal, an A-deficient and a B-normal. Further studies were conducted by Boyer *et al.* (1962). They noted that all Caucasian Americans were of type B, and that several Greek Glc-6PD-deficient subjects were of type B as well. Kirkman (1962a) reported almost identical findings, except that he called "type A" and "type B" as "fast" and "slow", respectively. Two Caucasians with CNHA were type B. The A-type enzymes from both normal and deficient people were partially purified, and compared for a number of different properties; they were found to be identical in all respects (Kirkman, 1959; Marks *et al.*, 1961).

A large number of variant forms of Glc-6PD have since been described. They have been named after the cities in which their possessors lived. Some differ in electrophoretic mobility from A and B while others do not. The latter must be distinguished on the basis of other properties, such as the K_m for Glc-6P and/or NADP, thermostability, affinity for 2-deoxyglucose-6P, pH optima, and so forth. Some of the variants are: Mediterranean (Kirkman *et al.*, 1964a), Tel-Hashomer (Ramot and Brok, 1964; Ramot *et al.*, 1964), Chicago I (Kirkman *et al.*, 1964b), Seattle (Shows *et al.*, 1964; Kirkman *et al.*, 1965), Ohio (Pinto *et al.*, 1966), Canton (McCurdy *et al.*, 1966), Kabyle (Kaplan *et al.*, 1967a and 1967b), Athens (Stamatoyannopoulos *et al.*, 1967), Kerala and West Bengal (Azevedo *et al.*, 1968), "New Guinea" variants (Kirkman *et al.*, 1968), Madrona (Hook *et al.*, 1968), Albuquerque and Duarte (Beutler *et al.*, 1968), Joliet I (Bowman *et al.*, 1969) and Hong Kong (Chan *et al.*, 1972). There are many additional variants. Reviews have been published periodically (Kirkman *et al.*, 1964c; Kirkman and Hanna, 1968; and Beutler, 1966).

By 1966, it was apparent that something ought to be done about standardizing the nomenclature of the many variants, and also about standardizing the methods used to characterize them, particularly because so many of them differ in kinetic and other properties. Most of them are not distinguishable by electrophoresis. An expert group was convened under the auspices of the World Health Organization to undertake this task (WHO Scientific Group on Standardization of Procedures for the Study of Glucose-6-Phosphate



Abbreviations: HK - Hexokinase GPx - Glutathione Peroxidase GR - Glutathione Reductase G6PD - Glucose-6-Phosphate Dehydrogenase PGM - Phosphoglucomutase PGD - 6-Phosphogluconate Dehydrogenase LDH - Lactate Dehydrogenase GSSG and GSH - Oxidized and Reduced Glutathione

Figure 33.1 Simplified Representation of Major Red Cell Pathways of Glucose Metabolism

Dehydrogenase, 1967; and World Health Organization, 1967). The nomenclature recommendations included retention of the designations "B", "A" and "A-" to indicate the usual forms of the enzyme, and the most common deficient A type seen in Black people. All other variants would be given trivial names after the city of origin. If there were more than one from a given city, arabic numbers would be used, e.g., Austin-1, Austin-2, etc. The genotype symbol for Glc-6-PD is *Gd*, and superscripts indicate specific genes, e.g. Gd^{A}, $Gd^{Mediterranean}$. Glc-6PD Mediterranean is the name assigned to the common deficient type with electrophoretic mobility identical to B. The activity level of the enzyme was to be designated as (−) 25% or less, (±) 25%–65%, (+) 65%–150% or (+ +) greater than 150% or 2 S.D. above the mean. These symbols could be incorporated into the phenotype designations if necessary, e.g., "Gd (+) B", "Gd (+) AB", "Gd (−) Canton", and so forth. Extensive standardized procedures were recommended for establishing the characteristics of variants. A complete discussion of the recommended testing procedures may be found in Motulsky and Yoshida (1969) as well.

A few more than 20 variants appeared in the 1967 WHO Technical Report. By 1971, the number had grown to almost 80, and a complete list was published by Yoshida *et al.* (1971). They placed the variants into one of five major classes: 1. Severe enzyme deficiency with chronic nonspherocytic hemolytic anemia; 2. Severe enzyme deficiency (< 10% normal); 3. Moderate to mild enzyme deficiency (10–60% normal); 4. Very mild or no enzyme deficiency (60–100% normal); and 5. Increased enzyme activity (greater than normal). In 1973, Beutler and Yoshida published a supplement to the 1971 list, containing over 25 more variants. Glc-6PD Long Prairie, which was in the supplemental list as an unpublished variant, is fully described by Johnson *et al.* (1977). Two more recent variants are Glc-6PD Ube (Nakashima *et al.*, 1977) and Glc-6PD

Velletri (Mandelli *et al.*, 1977). A recent list of variants was given by Beutler (1978). Undoubtedly, there are a number of other new ones. Well over a hundred variants have thus been described. The vast majority of them are rare. The only relatively common variants appear to be A(+) and A(−) which are common in Black people, "Markham" in New Guinea, "Taiwan-Hakka" in Hakka Chinese, "Union" in Filipinos, "Camplellur" in Pakistanis (about 2%), "Debrousse" in Arabs, "Athens" in Greeks, and "Mediterranean" which has been observed in Greeks, Sardinians, Sephardic Jews and Asiatic Indians. The A(+) and A(−) are the only readily distinguishable variants since they have a different electrophoretic mobility from the normal B. "Mediterranean" might be detected as a "B" with exceedingly low activity. Since Glc-6PD A(+) is nearly always associated with Blacks, it is worth noting that Angelopoulos and Delitheos (1970) described an A(+) in a Greek woman. Grunbaum *et al.* (1978b) found a small number of Glc-6PD A(+) among 5,916 California Caucasians in their population survey as well.

33.1.4 X-Linkage of the *Gd* locus—X-chromosome inactivation

In 1957, studies were carried out at Johns Hopkins on the primaquine sensitivity characteristic in 296 Black people, a number of whom belonged to 17 families. The GSH stability test was used to assess sensitivity, and three classes of individuals were seen. One was insensitive, another was sensitive and there was a class having intermediate sensitivity (Browne, 1957; Childs *et al.*, 1958). All the data suggested a mother to son mode of inheritance, characteristic of X-linkage. The gene, however, showed variable penetrance, and there was some variability within a phenotypic class with respect to GSH stability. Kirkman (1962a) and Boyer and Porter (1962) soon established that the electrophoretic A variant, common in Blacks, segregated as if it were controlled at an X-linked locus. It has also been firmly established that *Gd* is linked to the gene for hemophilia A, which is a firmly established X marker (Boyer and Graham, 1965).

For years it was thought that both the X chromosomes in females were genetically active. If they were, one would expect to find more gene product in XX individuals than in XY ones, and one does not. In *Drosophila*, there are "dosage compensation" mechanisms that can account for the observations. In the late 1950's it began to appear that the situation in mammals might be quite different. The chromatin body in XX cells was demonstrated by Ohno *et al.* (1959) to consist of heterochromatic chromosomal material derived from one of the X chromosomes. Since heterochromatin was known to be genetically inactive in some insect species, this finding raised the prospect that XX cells contain an inactive X chromosome. Additional aspects of this subject were discussed in section 1.2.4.4., and a little more is said in section 49.2.

In 1962, Beutler *et al.* established that the expression of Glc-6PD in human beings was consistent with the Lyon hypothesis, i.e., with inactivation of one of the X chromosomes in XX cells. Inactivation is thought to occur fairly early in the embryonic development of females, and to be random with respect to which of the X chromosomes becomes inactivated. As a result, it is to be expected that the fully developed female body will contain two different populations of cells, one descended from cells in which the maternally-derived X chromosome became inactivated, and the other from the cells in which the paternally-derived one did. In 1962, Beutler *et al.* presented evidence that the red cells of females heterozygous for Glc-6PD deficiency behave exactly as expected on the basis of two separate cell populations, one deficient and the other normal. Similar results were obtained by Davidson *et al.* (1963). In 1965, Gall *et al.* were able to prove the existence of two red cell populations in heterozygous females using tests on individual erythrocytes. Beutler (1969a) has pointed out that minor modification of the inactivation hypothesis can account for some of the "variable penetrance" seen in Glc-6PD deficiency syndromes. During the embryological and post-natal developmental stages, some natural selection could be occurring in the two cell populations, which might lead to unequal numbers of the two kinds of cells in the adult. The original 1:1 ratio could be significantly altered by quite mild selective forces, and these need not be related to Glc-6PD activity *per se*. If a gene favoring red cell production were present, for example, on the X chromosome having the normal *Gd* gene, then cells having this chromosome would proliferate much more readily than those having the inactive X. The end result would be an adult with near normal Glc-6PD levels, even though she was heterozygous for a deficiency gene. This interesting subject was well reviewed by Beutler (1968 and 1969a).

33.1.5 Biochemical studies on Glc-6PD

The earlier biochemical studies on the enzyme have been reviewed by Noltmann and Kuby (1963). The normal enzyme and that of several variant types have been purified extensively in a number of laboratories and characterized (Kirkman, 1962b; Kirkman and Hendrickson, 1962; Yoshida, 1966, 1967 and 1968; Yoshida *et al.*, 1967; and others). Glc-6PD appears to be a polymer, consisting of subunits of MW 55,000. Estimates of the MW of the "native" protein have varied (Chung and Langdon, 1963; Ratazzi, 1968), apparently because the enzyme can exist in several different states of aggregation. Bonsignore *et al.* (1971) studied the interconversion of the different forms. The monomer is catalytically inactive, but the dimer and tetramer are both active. The catalytically active enzyme contains bound NADP, and removal of this cofactor leads to dissociation with concomitant loss of activity (Kirkman and Hendrickson, 1962; Chung and Langdon, 1963; Bonsignore *et al.*, 1971). The active dimer has 18 -SH groups, and no disulfide bridges (Yoshida, 1973). Oxidation of the sulfhydryls reduces activity, reduces the K_m for 2-deoxy-Glc-6P, deamino-NADP and NAD, and increases the anodal electrophoretic mobility. Some 70% of the -SH groups may be oxidized, however, without observable

effect. The A(+) enzyme from common variant Negro subjects differs from the B enzyme by a single amino acid, there being an Asp in the A(+) in place of an Asn (Yoshida, 1967). The deficient Negro variant A(−) enzyme apparently represents a structural variant different from the A(+) (Yoshida *et al.*, 1967; Yoshida, 1968).

It has been known for some time that red cell stroma has an inactivating effect upon Glc-6PD (Carson *et al.*, 1959), believed to be related to the stabilizing effects of NADP upon the enzyme. The stroma appear to be able to deplete the enzyme of NADP, thus inactivating it (Carson *et al.*, 1966). It is routine, therefore, to add exogenous NADP to hemolysates and/or to electrophoretic gels in order to prevent this phenomenon.

33.1.6 Medicolegal applications

Glc-6-PD variant typing is apparently not often employed for purposes of disputed parentage testing. No published reports of such application were found.

The only discussion of Glc-6PD phenotyping in bloodstains was found in Culliford (1971) and the system was not enthusiastically recommended. Only the major electrophoretic variant in Blacks, the A(+), would be of much routine value. Common variant patterns are shown in Figure 33.3. Glc-6PD may be detected in gels following electrophoresis by the addition of substrate, NADP, $MgCl_2$, MTT and PMS in pH 8 buffer. Zones of activity appear as blue formazan bands where MTT is reduced by NADPH (Figure 33.2). The other variants require much more involved protocols for characterization. The recommendations of Culliford (1971) closely followed those of the WHO Technical Report, No. 366 (1967). Electrophoresis in 15% thin starch gels, containing 2 mg NADP per plate, in Tris-EDTA-boric acid buffers was found to be generally satisfactory, although Tris-HCl, ph 8.8, buffers were used as well. Procedures for Glc-6PD typing on various forms of cellulose acetate membranes have been devised by Rattazi *et al.* (1967) and Ellis and Alperin (1972). Grunbaum and Zajac (1976) described a CAM procedure applicable to hemolysates and to bloodstain extracts. Culliford (1971) noted that loss of activity of the enzyme is a serious problem, but that bloodstains up to 4 weeks old could be attempted, and would sometimes give results. Although the A(−) and A(−)B variants could be detected by electrophoresis in fresh blood by their deficiency of activity, diagnosis in bloodstains is an uncertain exercise. Losses of activity resulting from drying, aging or other effects are indistinguishable from low intrinsic activity. BA heterozygous patterns are observed only in women. The A variant will almost always be observed in Black people, although it needs to be remembered that Angelopoulos and Delitheos (1970) found an A(+) pattern in a Greek woman, and Grunbaum *et al.* (1978b) found 0.1% and 0.4% of 2,071 White women and 3,845 White men, respectively, in California to be Glc-6PD A(+).

Sperm cells show Glc-6-PD activity, and there is some activity in seminal plasma as well, but the cells have more activity on a per volume basis assuming 8×10^7 cells/mℓ semen (Blake and Sensabaugh, 1976 and 1978). The activity in whole semen is only about 2% of that in whole blood, however. Blake and Sensabaugh (1978) estimated that the minimum amount of whole semen which would be required for Glc-6-PD determination would be 40 μℓ, and even more with seminal plasma alone. The comparable figure for whole blood was 1 μℓ.

33.1.7 The distribution of common *Gd* phenotypes in U.S. Black populations

There are not many U.S. population studies in which information about the frequency of the electrophoretic variants has been collected. The data are given in Table 33.1.

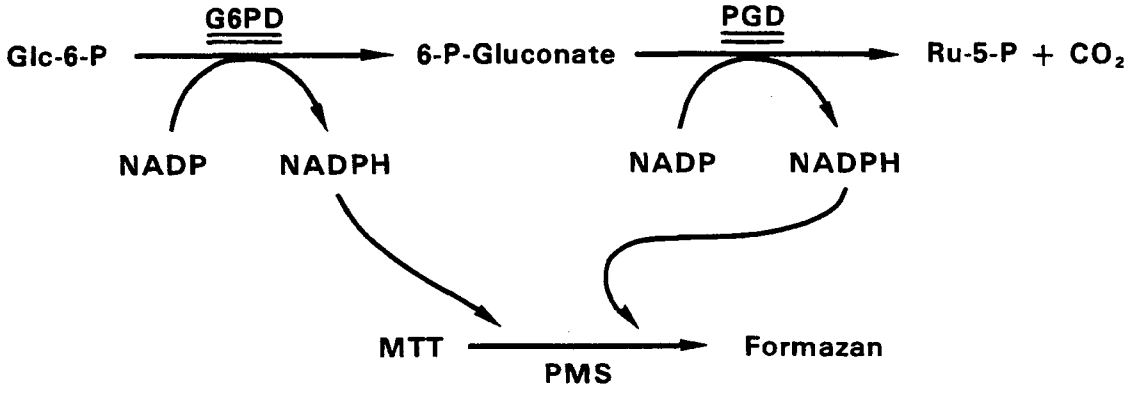


Figure 33.2 Basis of Detection Reaction Sequences for Glc6PD and PGD

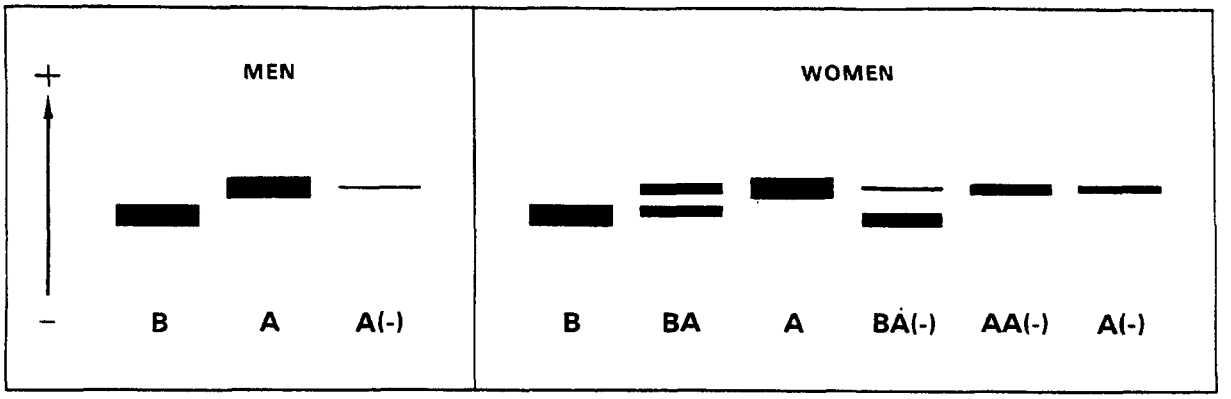


Figure 33.3 Electrophoretic Patterns of Glc-6PD Common Variant Isoenzymes

33.2 6–Phosphogluconate Dehydrogenase [PGD; 6–PGD; Phosphogluconate Dehydrogenase (decarboxylating); 6–Phospho–D–Gluconate: $NADP^+$ 2–Oxidoreductase (decarboxylating); E.C. 1.1.1.44]

33.2.1 Recognition of PGD

In connection with their pioneering studies on Glc-6-PD, discussed in section 33.1.1, Warburg *et al.* (1935) and Warburg and Christian (1936) realized that the oxidation of Glc-6P proceeded beyond 6-P-gluconate in yeast extracts. They, and Dickens (1936 and 1938), demonstrated that the further breakdown of 6-P-gluconate consisted of an oxidative decarboxylation reaction leading to a pentose-P, which could in turn be further metabolized to yield a complex mixture of products. NADP was shown to be a required co-factor for the reaction. The identity of the pentose phosphate product was not established for quite some time. Dickens (1938) thought that it was ribose-5-P, but this supposition was later shown to be wrong. Strong indications that the reaction product was ribulose-5P (Ru5P), and not ribose-5P (R5P), came from the work of Horecker and Smyrniotis (1950 and 1951) and of Horecker *et al.* (1951) with fairly pure PGD preparations from yeast. They were quite certain that Ru5P was the product of the PGD reaction, but that contamination of the preparation by pentose phosphate isomerase, which catalyzes the Ru5P $\rightleftarrows$ R5P interconversion, was leading to a rapid equilibrium in favor of the aldopentose-P. The equilibrium of the pentose phosphate isomerase reaction lies very far in the direction of R5P. Final proof of this idea came when Pontremoli *et al.* (1961) obtained a pure, crystalline preparation of PGD, free of PPI. PGD is the second enzyme in the hexose monophosphate pathway (section 33.1.2; Figure 33.1), and the earlier biochemical studies on the enzyme were reviewed by Noltmann and Kuby (1963).

33.2.2 Genetic variation of PGD

There are quite a number of PGD variants known, many of them quite rare. Some of the variant alleles give rise to isozymes which have normal levels of activity. These are discussed in section 32.2.2.1 in the order of their discovery. Other named phenotypic variants of PGD exhibit deficiency in activity. These are presented in section 32.2.2.2. A third class of PGD variant could be constructed, namely those in which the electrophoretic patterns are significantly different in the presence and absence of certain compounds. This class would have two members, PGD Friendship and PGD Natal. These have been included in section 32.2.2.1. The nomenclature history of PGD variants is quite confusing in certain respects (section 32.2.2.3), and it may be helpful to consult Table 33.2 while reading through section 32.2.2. Genetic variation of PGD is reviewed by Brewer (1969) and by Beckman (1978).

32.2.2.1 Variants exhibiting normal activity. In 1963, Fildes and Paar described the first electrophoretic variants of PGD. Electrophoresis at 10V/cm on starch gels in phosphate buffer, pH 7, revealed two electrophoretic types. Most people showed a single "A" band, but in 10 out of 150, a pattern with the "A" band and a slightly slower (more cathodal) "B" band was seen. Investigations of several families indicated that the variant pattern was the expression of heterozygosity at a simple autosomal locus. Individuals having the variant pattern showed no alteration in red cell PGD activity. As the electrophoretic procedures improved, Fildes and Paar realized that the heterozygote pattern also had a very weak "C" band, slower than the B band. This observation was first reported at the VI International Congress of Biochemistry in 1964, and was explained by Paar (1966). Bowman *et al.* (1966) examined hemolysates from 1,016 Americans, and confirmed the findings of Fildes and Paar (1963). They found a homozygous variant person in their survey as well. Bowman *et al.* (1966) suggested that the phenotypes be designated A, A-B and B, and the responsible alleles as Pd^A and Pd^B. Family studies fully sup-

Table 33.1 Distribution of Common *Gd* Phenotypes in U.S. Black Populations

		Phenotypic Frequency — Number (Percent)						
		MALES		FEMALES				
Population	Total	B	A★	B	A★	BA ☆	Gd^{A} ⊙	Reference
Baltimore, MD	311	206 (66.2)	105 (33.8)				0.34	Boyer et al., 1962
	100			52 (52)	13 (13)	35 (35)	0.31	
Oklahoma	135	88 (65.2)	47 (34.8)				0.34	Kirkman and Hendrickson, 1963
	39			23 (59.0)	3 (7.7)	13 (33.3)	0.24	
Chicago, IL	35	28 (80)	7 (20)				0.20	Shih and Hsia, 1969
	65			45 (69.2)	9 (13.8)	11 (16.9)	0.22	
California	896	(73.0)	(26.7)				0.270	Grunbaum et al., 1978b
	111			(63.1)	(9.9)	(23.4)	0.224	
Detroit, MI	252	173 (68.7)	78 (31.0)				0.31	Stolorow et al., 1979 and see Shaler, 1978

★ Includes A(-) ☆ Includes BA(-) ⊙ Gene frequency of Gd^{A} and Gd^{A^-} combined

ported the two allele mechanism of inheritance. Paar (1966) described several additional variants that had been found, some of which were more fully described in later papers. One of these showed a "fast" triplet pattern, while another showed a pattern quite similar but not identical to the common variant and had a weak A band and a weak C′ band, with a strong B′ band. The designations B′ and C′ were used to distinguish the bands in these variants from those in the common variant, the mobilities being just slightly different. Two homozygotes for the allele determining the usual variant had been seen. By 1966, the variants were acquiring names. The names were often used for the phenotypes, and, by analogy to the Glc-6PD variants, were those of the places where the possessors lived. The common variant, which had first indicated to Fildes and Paar (1963) that PGD was polymorphic, and which Brewer *et al.* (1966) called A-B, was still called the "common variant" by Paar. The homozygote for the common variant gene was named the "Canning variant". Bowman *et al.* (1966) called it "B". The fast triplet pattern was named "Richmond", and was more fully described by Carter *et al.* (1968). The other rare pattern, a triplet with weak A and C′ bands, but a strong B′, was called "Hackney", and it, too, was more fully described by Carter *et al.* (1968). Paar and collaborators had also proposed a series of Greek letter superscripts for the alleles, using "PGD" as the locus symbol. All the variants mentioned thus far, except Canning, were heterozygotes of the usual allele. Thus, the common type was genotypically $PGD^{\alpha}PGD^{\alpha}$, the common variant was $PGD^{\alpha}PGD^{\beta}$. The Canning $PGD^{\beta}PGD^{\beta}$, the Richmond $PGD^{\alpha}PGD^{\gamma}$, and the Hackney $PGD^{\alpha}PGD^{\delta}$. In 1967, Davidson described an additional mutant type, called "Friendship". Family studies indicated that it represented heterozygosity of the Friendship allele with the normal allele. "Friendship" is peculiar, in that it was detected in gels from which NADP had been excluded. Some workers routinely add NADP to their gels (primarily to stabilize the Glc-6PD which may be simultaneously determined), while others do not. This matter is discussed below. If "Friendship" is run in an NADP-containing gel, however, it exhibits the pattern of a usual PGD. Further, leucocytes from the original "Friendship" propositus show only the fastest band of the red cell pattern. Davidson (1967) said, therefore, that "Friendship" should be diagnosed by electrophoretic runs in both NADP-containing and NADP-less gels, and by comparing red cell and white cell patterns. In 1968, Tuchinda *et al.* described a variation in several unrelated people in Thailand. The variant pattern resembled that of "Richmond" and was called "PGD-Thai". It was not the same as the Friendship variant. It is possible that it is the same as Richmond. Blake and Kirk (1969 found a new variant in Australian aborgines, which occurred in polymorphic frequencies in these people,

especially in the Elcho Islands. This phenotype was called PGD Elcho. An Elcho phenotype has recently been observed in Finland (Virtaranta-Knowles and Nevanlinna, 1979). Tariverdian *et al.* (1970b) found a variant phenotype in a German population sample which looked very much like "Friendship" electrophoretically, but its pattern was not altered by the addition of NADP to the gel. The gene for this variant was segregating in two families, and the phenotype was called PGD–Freiburg. In 1973, Blake *et al.* found a new phenotype in a Chinese resident of Singapore, which was named PGD–Singapore. The allele codes for isoenzymes which run cathodic to (slower than) the usual A on electrophoretic gels (Blake *et al.*, 1973b). Blake *et al.* (1974) carried out an extensive population survey, and discovered six new PGD alleles. All represented heterozygous expression of unusual alleles in combination with the usual one, and the variants were called Wantoat, Canberra, Kadar, Caspian, Bombay and Natal. Wantoat and Canberra were slow electrophoretic variants, while the others were fast, relative to PGD A. Natal is a peculiar variant. Treatment of Natal samples with mercaptoethanol causes them to change to a form which looks "usual" electrophoretically. Blake *et al.* (1974) found a homozygote for the Kadar variant, as well as a Kadar/C heterozygote and a Natal/C heterozygote. In 1974, Jenkins and Nurse surveyed 34 distinct populations in southern Africa, and found a further PGD variant with a slow electrophoretic pattern. This was called PGD Oshakati.

32.2.2.2 Variants exhibiting deficiencies in activity—PGD^o and PGD^w. In 1964, inherited PGD deficiencies were observed independently in this country and in England. Brewer and Dern (1964a and 1964b) observed a Black woman whose red cells showed about 50% the normal PGD activity. Extensive studies of her family revealed a large kindred in which the 50% PGD activity condition was segregating in four generations. The most logical genetic explanation was heterozygosity in the affected members for the usual allele and an inactive one. This pedigree is quite an interesting one, because Glc-6PD deficiency was also segregating in this family. Paar and Fitch (1964) found a man in London with about 50% normal PGD activity. This condition was found in members of four generations of his family. The propositus was extraordinary, because his electrophoretic pattern was that of a homozygous Canning variant, and his genotype was imagined to be $PGD^{\delta}PGD^o$ in the old terminology (PGD^CPGD^o in the newer terminology—see below). His phenotype was named the "Half-Canning" for the time being. Dern *et al.* (1966) extended the earlier population studies of Brewer and Dern (1964a and 1964b), and found quantitative variants in a number of families. In several, the affected people showed 50% of normal activity, while in others, the deficiency did not appear to be fully expressed. The latter had greater than 50% of the usual activity. One person had the common variant type (according to the nomenclature of Paar; the A/B phenotype, according to the nomenclature of the authors), and both bands of activity were 50% reduced. The result suggested a mutation in a regulatory gene, but this could not be proven in the particular family. In 1967, Paar and Fitch reported several additional phenotypes in the growing English population sample and summarized the studies that had been carried out thus far. In this paper, the newer nomenclature also appears for the first time (see in section 32.2.2.3). The common type was given the genotypic designation PGD^APGD^A. The Canning variant allele was designated PGD^C. The common variant would thus be PGD^APGD^C while the homozygous Canning variant would be PGD^CPGD^C. The phenotype previously called "Half-Canning", PGD^CPGD^o, was re-named the "Newham" type. An electrophoretically usual type, with 50% normal activity, was reported. The probable genotype was PGD^APGD^o, and this was called "Ilford". The 50% activity subjects seen by Dern *et al.* (1966) are probably "Ilford" types. The London family in which the PGD^o-like allele was segregating was fully described by Paar and Fitch (1967). Another English family was described in this paper in which two fully deficient individuals, a brother and sister, occurred. These individuals were mentioned by Paar (1966). The activity assays and genetic data indicated that these people were not homozygotes for PGD^o, but rather that the deficiency had a different genetic basis. The fully deficient phenotype was called "Whitechapel" and the genotype denoted PGD^WPGD^W. Heterozygotes for this allele and PGD^A, i.e. the PGD^APGD^W types, were called "Dalston" and they have about 75% of normal PGD activity, while the Ilford types have about half. The people in the sample of Dern *et al.* (1966) who had more than 50% enzyme activity, but less than or about 75% activity, may well have been Dalston phenotypes (Brewer, 1969).

Paar and Fitch (1967) raised an important point in connection with the assessment of these deficiency phenotypes. Enzyme activity in the red cell must be expressed in terms of some basis that reflects the amount of blood, or the number of red cells, for which the value is quoted. Units of activity alone are quite meaningless. A common way of expressing activity is in terms of "per gram Hb". Under some circumstances, PGD activity per g Hb will be quite satisfactory, but Paar and Fitch (1967) pointed out that if one of the individuals being compared is anemic, and has a low Hb concentration, the PGD activity expressed as Units/g Hb might turn out to be quite misleading. It is good practice, therefore, to assay another enzyme (in the case of PGD, the other one is usually Glc-6PD), and express the activity of the enzyme under study and the reference enzyme as ratios. In the case of PGD, the PGD/Glc-6PD ratio would correctly reflect a deficiency in an anemic person which a PGD activity in Units/g Hb might fail to disclose.

33.2.2.3 The nomenclature of PGD variants. The PGD variant nomenclature is somewhat confusing, but most of the time the designations are clear. Table 33.2 gives a summary of the variants. The electrophoretic patterns in the table are not really comparable to one another, since many of them were found in different laboratories, and everyone does not employ the same electrophoretic conditions. The

Table 33.2 Nomenclature and Properties of PGD Variants

Phenotype Designation	Previous Phenotype Designation(s)	Genotype Designations: Previous[1]	Genotype Designations: Current	Electrophoretic Mobility Relative to PGD-A[2]	PGD Activity % of Usual	Reference(s)
Usual; Type A	Normal; Type I	$PGD^{A}PGD^{A}$	$PGD^{A}\,PGD^{A}$[3]; $Pd^{A}\,Pd^{A}$[4]		100	–
Common Variant; Type AB[4]	Type II	$PGD^{A}PGD^{B}$	$PGD^{A}\,PGD^{C}$[3]; $Pd^{A}\,Pd^{B}$[4]		80-100	Fildes and Parr (1963); Bowman et al. (1966)
Canning; Type B[4]	Type III	$PGD^{B}PGD^{B}$	$PGD^{C}\,PGD^{C}$[3]; $Pd^{B}\,Pd^{B}$		70-90	Bowman et al. (1966); Parr (1966)
Newham	Half Canning; Type VII	$PGD^{B}PGD^{0}$	$PGD^{C}\,PGD^{0}$		40-50	Parr and Fitch (1964 and 1967)
Richmond	Richmond; Type IV	$PGD^{A}PGD^{Y}$	$PGD^{A}\,PGD^{R}$		100	Parr (1966); Davidson (1967)
Hackney	Hackney; Type V	$PGD^{A}PGD^{J}$	$PGD^{A}\,PGD^{Hackney}$; $PGD^{A}\,PGD^{N}$		100	Parr (1966)
Ilford	Half-Activity; Type VI	$PGD^{A}PGD^{C}$	$PGD^{A}\,PGD^{0}$		50-60	Parr (1966); Brewer and Dern (1964a and 1964b); Dern et al. (1966)
Fully Deficient	Fully Deficient; Type VIII	$PGD^{0}PGD^{0}$	$PGD^{0}\,PGD^{0}$		–	Not observed
Whitechapel	Fully Deficient		$PGD^{Y}\,PGD^{Y}$		3%	Parr (1966); Parr and Fitch (1967)
Dalston			$PGD^{A}\,PGD^{Y}$		75%	Dern et al. (1966); Parr and Fitch (1967)
Friendship	Friendship		$PGD^{A}\,PGD^{Friendship}$; $PGD^{A}\,PGD^{F}$	+NADP / −NADP	100	Davidson (1967); Brinkman (1971)
Thai[5]			$PGD^{A}\,PGD^{Thai}$	like Richmond	100	Tuchinda et al. (1968)
Elcho			$PGD^{A}\,PGD^{Elcho}$		100	Blake and Kirk (1969)
Freiburg			$PGD^{A}\,PGD^{Freiburg}$; $PGD^{A}\,PGD^{F}$	±NADP	100	Tariverdian et al. (1970b); Brinkman (1971)
Singapore			$PGD^{A}\,PGD^{1}$		100	Blake et al. (1973b)
Wantoat			$PGD^{A}\,PGD^{Wantoat}$		100	Blake et al. (1974)
Canberra			$PGD^{A}\,PGD^{Canberra}$		100	Blake et al. (1974)
Kadar			$PGD^{A}\,PGD^{Kadar}$		100	Blake et al. (1974)
Caspian			$PGD^{A}\,PGD^{Caspian}$		100	Blake et al. (1974)
Bombay			$PGD^{A}\,PGD^{Bombay}$		100	Blake et al. (1974)
Natal			$PGD^{A}\,PGD^{Natal}$	−CH_2CH_2SH / +CH_2CH_2SH	100	Blake et al. (1974)
Oshakati			$PGD^{A}\,PGD^{Oshakati}$; $PGD^{A}\,PGD^{1}$		100	Jenkins and Nurse (1974)

[1] According to the earlier papers of Parr and collaborators

[2] Patterns are given only to indicate the relative mobilities of variant isozymes and PGD-A; the types have not all been run under the same conditions, and, as such, are not directly comparable to one another

[3] Revised nomenclature according to Parr and Fitch (1967) and Davidson (1967)

[4] Nomenclature according to Brewer, Dern and collaborators

[5] May be equivalent to Richmond; Jenkins and Nurse (1974) said it may be equivalent to Elcho

representations have been constructed from published photographs and drawings.

The only variant that is at all common is the "common variant". The genotype of such persons is now generally denoted $PGD^{A}PGD^{C}$, and the phenotype is usually denoted as "AC". Some workers employ the designations of Bowman *et al.* (1966), in which the common variant allele is denoted Pd^{B}. The genotype of the common variant heterozygote is thus $Pd^{A}Pd^{B}$, and the phenotype is called "AB". Both the "Freiburg" and the "Friendship" alleles have been denoted PGD^{F}, and Brinkmann (1971) suggested that they might be distinguished by using PGD^{F} and $PGD^{F'}$ for Friendship and Freiburg, respectively. It is probably best to use the complete name if there is any chance of confusion. The majority of variants are very rare, although some occur in appreciable frequencies in certain isolated populations.

32.2.2.4 Linkage relations and chromosomal localization of PGD. The data of Weikamp *et al.* (1970) indicated a linkage between *PGD* and *Rh* loci. Renwick (1971a) reported that *PGD* was syntenic to *Rh* and PGM_1 loci, thus localizing it on chromosome 1. This finding has been confirmed (Westerveld and Meera Khan, 1972; Jongsma *et al.*, 1973; Douglas *et al.*, 1973).

33.2.3 Biochemical studies on PGD

The earlier studies on PGD have been reviewed by Noltmann and Kuby (1963). Not too much detailed biochemical work appears to have been carried out on purified red cell preparations. Paar and his collaborators, in the earlier papers, thought that the active enzyme was probably a dimer, and that at least PGD^{A} and PGD^{C} were coding for different monomers. Bowman *et al.* (1966) did not completely accept this idea. Some of the variant enzymes show variable thermal denaturability as well as variable denaturability in the presence of urea or iodoacetamide, the PGD-A enzymes being more stable than the PGD-C (Paar and Paar, 1965; Carter *et al.*, 1966 and 1968). The PGD-A enzyme did not differ in these respects from Hackney and Richmond, however (Carter *et al.*, 1968). Shih *et al.* (1968b) partially purified PGD from A, AC and C red cells. The A and C forms differed in their K_m for 6-phosphogluconate but not for NADP. Pearse and Rosemeyer (1974) may have obtained the purest preparation of PGD from red cells. Their preparation was homogeneous in the ultracentrifuge, but showed some heterogeneity upon starch gel electrophoresis. Some of the properties of the purified enzyme were described by these workers, but they were not concerned with genetic variants of PGD. The MW of PGD is about 80,000 (Kazazian, 1966).

Carson *et al.* (1966) discovered that incubation of PGD with NADP in the presence of red cell stroma resulted in a significant loss of enzyme activity. Neither stroma, nor NADP alone, caused the effect. Subsequent studies by this group (Ajmar *et al.*, 1968) revealed that the stroma-NADP inactivation effect is an indirect one. Stroma contains an NAD(P)-ase. The designation "NAD(P)-ase" means that the enzyme will act on NAD or on NADP. The enzyme acts on NADP to produce, as one of the products, 2′-phosphoadenosine diphosphate ribose (P-ADPR). This molecule modifies PGD in some way leading to inactivation. If hemolysate, containing stroma, is incubated with NADP for increasing periods of time, and the electrophoretic mobility of PGD examined as a function of time of incubation, the PGD-A band is seen to become weaker with the simultaneous appearance of a slower band. The final pattern greatly resembles that seen with a homozygous Canning variant. It should be noted that this effect of NADP is quite opposite to the stabilizing influence this cofactor exerts on Glc-6PD (section 33.1.5).

33.2.4 Medicolegal applications

The PGD polymorphism is utilized in a number of laboratories in cases of disputed parentage. Since the frequency of the PGD^{C} allele does not reach very high frequencies in most populations, PGD is not one of the more powerful systems for the exclusion of true nonfathers. In German populations, the system may be expected to exclude 2%-3% of falsely accused fathers (Brinkmann, 1971). In England, the figure is 2.5% (Boorman *et al.*, 1977). In this country, the values are about 2.3% for Caucasians and 3.3% for Blacks (Polesky *et al.*, 1976; Dykes and Polesky, 1978). In the more recent work, the figure for Caucasians is given as 1.8%, and is probably based upon gene frequencies calculated for a larger population sample. PGD isozymes are fully developed in fetal blood (Chen *et al.*, 1977).

PGD isozymes have been determined in bloodstains. Procedures for typing the enzyme have been described by Culliford (1971) and by Brinkmann (1971). The methods differ only slightly. Brinkmann (1971) utilized phosphate buffers at pH 6.8 and thicker starch gels, where Culliford (1971) recommended the use of thin starch gels, and a slightly different buffer concentration. Except for slight differences in the pH of buffers, these procedures closely resemble the original method used by Fildes and Paar (1963). PGD isozymes are detected in gels after electrophoresis by the addition of an overlay of substrate, NADP, $MgCl_2$, PMS and MTT in pH buffer, and looking for formazan bands (Figure 33.2). NADP in pre-treating solutions or incorporated into gel buffers is to be scrupulously avoided with bloodstains, since the stroma are still present. Carter *et al.* (1968) noted that additional weak bands, anodal to PGD-A, may appear if NADP is present, and Culliford (1971) confirmed this finding. These same bands may be seen in old lysates (Culliford, 1971). PGD is quite labile in bloodstains. Brinkmann (1971) said that he could differentiate A and AC types in stains kept at room temperature for up to four weeks. Welch (1972b) could detect the phenotypes for up to 15 days in dried bloodstains. Henke (1979) recently recommended caution in the interpretation of PGD patterns, especially where unusual variants are involved. These variants can exhibit variability with respect to the retention of the pattern characteristics, even upon freezing and thawing.

Other electrophoretic procedures have been described for PGD phenotyping (see in Brewer, 1969 and 1970). Sonneborn (1972) described a cellulose acetate membrane technique. A number of procedures have been devised for the simultaneous determination of PGD along with other isozyme systems: with AK (Brinkmann and Thoma, 1970); with AK and ADA in lysates and bloodstains (Brinkmann and Dirks, 1971); with GPT, PGM and AK (Goedde and Benkmann, 1972); and with ACP on thin layer agarose gels (Martin and Voss, 1978).

Suyama and Imai (1975) reported that PGD types could be determined from tooth parts, and Oya *et al.* (1978) reported successful PGD typing in human hair bulbs up to two weeks after the hair was extracted.

PGD isozymes occur in sperm cells, and the type is the same as that seen in red cells (Brinkmann and Koops, 1971; Blake and Sensabaugh, 1976). The concentration of PGD is much lower in seminal plasma, however (Culliford, 1971; Blake and Sansabaugh, 1976 and 1978). Whole semen contains only about 4% of the PGD activity seen in whole blood on a per volume basis, and it was estimated that a minimum quantity of 25$\mu\ell$ semen would be needed for PGD determination, as against about 1$\mu\ell$ for blood (Blake and Sensabaugh, 1978).

Gibbs (1968) reported that PGD is present in "vaginal fluid", but the activity levels observed were not compared with those in blood, nor were they referenced to a defined amount of "vaginal fluid". The studies were undertaken to explore a possible relationship between PGD levels in vaginal fluid and cervical carcinoma.

33.2.5 The distribution of PGD phenotypes in U.S. populations

The distribution of PGD variants for world populations was reviewed by Tills *et al.* (1970b). A ten-fold decimal error, causing the frequencies to be 10 times too large, was subsequently corrected (Tills *et al.* 1971b). The frequency of PGD^C is low in most Caucasian populations. It can reach much higher levels (10–20%) in some Asian Indian, African and Middle Eastern populations. The data for U.S. populations appears in Table 33.3.

Table 33.3 Distribution of PGD Phenotype in U.S. Populations

Population	Total	Frequency of Occurrence — Number (Percent) A	AC	C	PGD^{A} ★	Reference
CAUCASIAN						
Unspecified locale	58	57 (98.3)	1 (1.7)		0.99	Dern et al., 1966
Unspecified locale	600	554 (92.3)	45 (7.5)		0.961	Bowman et al., 1966
Buffalo, NY	1,377	1,313 (95.3)	62 (4.5)	2 (0.2)	0.976	Davidson, 1967
Chicago, IL	101	97 (96)	4 (4)		0.98	Shih and Hsia, 1969
Seattle, WA	647	624 (96.5)	22 (3.4)	1 (0.1)	0.98	Giblett, 1969
California	4,472	(96.2)	(3.7)		0.98	Grunbaum et al., 1978b
Detroit, MI	503	482 (95.8)	20 (4.0)	1 (0.2)	0.978	Stolorow et al., 1979 and see Shaler, 1978
NEGRO						
Unspecified locale	296	278 (93.9)	18 (6.1)		0.97	*Dern et al., 1966*
Unspecified locale	416	385 (92.5)	31 (7.5)		0.963	Bowman et al., 1966
Buffalo, NY	1,226	1,141 (93.0)	83 (6.8)	2 (0.2)	0.964	Davidson, 1967
Chicago, IL	101	93 (92.1)	8 (7.9)		0.96	Shih and Hsia, 1969
Seattle, WA	506	452 (89.3)	52 (10.3)	2 (0.4)	0.945	Giblett, 1969
California	787	(92.6)	(7.2)	(0.4)	0.964	Grunbaum et al., 1978b
Detroit, MI	504	462 (91.7)	39 (7.7)	2 (0.4)	0.955	Stolorow et al., 1979 and see Shaler, 1978

★ Gene frequency

SECTION 34. GLYOXALASE I

34.1 Recognition of Glyoxalase

Glyoxalase I [GLO;. GLO I; Lactoyl-glutathione lyase; S-lactoyl-glutathione methylglyoxal-lyase (isomerizing); E.C. 4.4.1.5] catalyzes the reversible conversion of reduced glutathione and methyl glyoxal to S-lactoylglutathione. In conjunction with its companion enzyme, glyoxalase II (GLO II; hydroxyacylglutathione hydrolase; S-2-hydroxyacylglutathione hydrolase; E.C. 3.1.2.6) which catalyzes the hydrolytic conversion of S-2-hydroxyacyl glutathione compounds to glutathione and the corresponding 2-hydroxy acid, the overall conversion of methyl glyoxal to lactate can be effected.

The presence of an enzyme activity in animal tissues which catalyzed the conversion of methyl glyoxal to lactate, and of phenyl glyoxal to mandelic acid, was simultaneously recognized by Neuberg (1913) and by Dakin and Dudley (1913a). The latter authors are responsible for the name "glyoxalase" (Dakin and Dudley, 1913b), and also noted that the enzyme was present in the red cells but not in the serum of dog blood. In 1932, Lohmann established that reduced glutathione was required for glyoxalase activity. Jowett and Quastel (1933) found that human red cells contain substantial GLO activity, and proposed that S-lactoylglutathione was an intermediate in the reaction leading to lactate. Yamazoye (1936) demonstrated the formation of the glutathione-methyl glyoxal adduct in crude liver extracts. In 1948, Hopkins and Morgan obtained evidence from partially purified beef heart preparations that a protein "factor" accelerated the formation of lactate from the methyl glyoxal and glutathione in the presence of glyoxalase. In 1951, Racker demonstrated that yeast contains two glyoxalases, which he called I and II. Glyoxalase I catalyzed formation of the S-lactoyl glutathione, and glyoxalase II catalyzed its hydrolytic breakdown into lactate and reduced glutathione. A further description of the methods used to study yeast glyoxalases was given by Racker in 1955. Crook and Law (1952) showed that beef heart contains both glyoxalases as well. Human erythrocytes contain substantial amounts of glyoxalase I, but no glyoxalase II (Cohen and Sober, 1945; Valentine and Tanaka, 1961; Paar *et al.*, 1977). Glyoxalase enzymes are widely distributed in nature.

34.2 Genetic Variation of Glyoxalase I

GLO polymorphism is a relatively recent finding. In 1975, Kömpf *et al.* in Germany, and Bagster and Paar in England, independently discovered that GLO exhibited genetically determined electrophoretic variation in red cells. Three phenotypes were observed, and were called "1", "2-1" and "2" by the German workers, and "slow", "intermediate" and "fast", respectively, by the English investigators. These could be explained by a pair of codominant autosomal alleles, and family and population studies were consistent with this idea (Kömpf *et al.*, 1975; Kömpf and Bissbort, 1975; Bagster *et al.*, 1975; Paar *et al.*, 1977). The alleles determining the common phenotypes are now denoted GLO^1 and GLO^2. The GLO^1 frequencies in Britain and Germany were very similar, 0.42 to 0.43. In Gambians, the GLO^1 was about 0.25 (Bagster *et al.*, 1975).

Additional population and family studies have been consistent with the codominant autosomal allelic pair hypothesis of inheritance (Olaisen *et al.*, 1976b; Martin and Ott, 1976; Meera Khan and Doppert, 1976; Brinkmann and Püschel, 1978). Ranzani *et al.* (1979) reported a new phenotype of GLO, which could be attributed to heterozygosity of GLO^2 with a new allele, GLO^3. There is, additionally, a silent allele, GLO^0. Olaisen *et al.* (1976b) mentioned that they had found a woman with no detectable GLO activity, but further studies could not be pursued. Rittner and Weber (1978) found a family, however, in which a GLO^0 allele was segregating in three generations. An unusual HLA haplotype was also segregating in this family, making it even more interesting, particularly in view of the established *GLO:HLA* linkage. Rubinstein and Suciu-Foca (1979) reported a GLO^0 allele segregating in three generations of a family. The *GLO* locus has been mapped on chromosome 6, and is linked to *HLA* and PGM_3. *GLO* is thought to lie between the PGM_3 and *HLA-B* loci (Olaisen *et al.*, 1976a; Weitkamp, 1976; Pretorius *et al.*, 1976). The electrophoretic patterns of GLO isozymes are shown diagrammatically in Figure 34.1.

34.3 Assay and Detection Methods for GLO

Assay procedures for glyoxalases were given by Racker (1955). These are applicable to the assay of red cell GLO. Spectrophotometric assay of GLO is based upon the fact that S-lactoyl glutathione, the product of the GLO I reaction, absorbs light of 240 nm. GLO I can thus be assayed by following the increase in absorbance at 240 nm, while GLO II can be assayed by following the A_{240} decrease (Racker, 1951 and 1952). GLO I can be assayed in crude hemolysates utilizing this principle (Paar *et al.*, 1977).

A number of different detection procedures for GLO isozymes in gels have been employed. Apart from its characteristic UV absorption, the product of the GLO I reaction has no other properties that make it readily detectable. The impracticality of spectrophotometric detection procedures in gels has prompted the employment of other procedures. The methods used have taken advantage of the fact that reduced glutathione occurs everywhere in the gel except at the sites of GLO activity. Two procedures, based upon the color reactions of unreacted GSH have been devised. In one

of these, MTT tetrazolium salt is dissolved in agar gel in the presence of either PMS (Bagster and Paar, 1975), or of DCPIP (Kömpf *et al.*, 1975), and overlaid on the electrophoretic gels. This overlay may contain the GLO substrates, or may be added after incubation of the electrophoretic gel with a filter paper overlay containing the substrates. Sites of activity appear as clear zones on an otherwise violet (formazan) background. In the second, introduced by Paar *et al.* (1977), the gel is overlaid with the GLO substrates on filter paper, and incubated to allow the reaction to occur. It is then overlaid with an iodine solution in agar gel. The unreacted glutathione in the electrophoretic gel reduces the iodine to iodide except at the sites of GLO activity, where the iodine reacts with the starch in the electrophoretic gel giving the familiar deep blue color. This procedure is applicable only to electrophoretic gels made from starch or containing it. Another procedure, used by Bagster and Paar (1975), is to add the substrates along with exogenous GLO II, LDH, NAD, PMS and MTT to the gel overlay. The GLO II catalyzes the formation of lactate from S-lactoyl glutathione at the sites of GLO I activity, and the lactate is oxidized to pyruvate by LDH with the concomitant reduction of NAD to NADH. The NADH then reduces MTT tetrazolium to formazan in the presence of PMS. The detection schemes are summarized in Figure 34.2.

34.4 Biochemical Studies on Glyoxalase I

No reviews of glyoxalase biochemistry were found. The earlier studies were mentioned in section 34.1. The first glyoxalase purified was a yeast enzyme (Racker, 1951). Glyoxalases from animal sources were not purified and characterized until relatively recently. Davis and Williams (1966) obtained relatively pure preparations from calf liver and from yeast. Their yeast enzyme was purified to a greater extent than was Racker's in 1951. More recently, affinity chromatography steps have been introduced into purification procedures, and very pure preparations have been obtained from pig erythrocytes (Mannervik *et al.*, 1972; Aronnson and Mannervik, 1977; Aronnson *et al.*, 1978), rat erythrocytes (Han *et al.*, 1977), and the livers of mice (Kester and Norton, 1975), rabbits (Elango *et al.*, 1978) and sheep (Uotila and Koivusalo, 1975). The yeast enzyme, and that from human red cells, have also been purified (Aronnson *et al.*, 1978). Most mammalian GLO I appear to be dimers, with MW about 43,000 to 48,000. The pig red cell enzyme exhibits two components when pure, and treatment with reduced glutathione converts them to a single form, suggesting that one of the "forms" is a mixed disulfide with GSH (Aronnson and Mannervik, 1977). This enzyme contains two -SH per mole. Davis and Williams (1966) found that the enzyme was inactivated by EDTA, but that activity could be more or less restored by incubation with Mg^{++} or Mn^{++}. The yeast enzyme was similarly inactivated, but its activity could not be restored by Mg^{++}. Atomic absorption analysis of the yeast enzyme detected Mg^{++}, and they concluded that this was a Mg^{++}-enzyme. All the mammalian

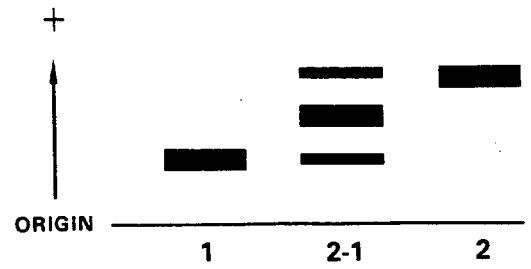


Figure 34.1 Electrophoretic Patterns of GLO Phenotypes

enzymes are inactivated by chelating agents as well, but activity can be restored at least to some extent by the addition of divalent metal ions, Mg^{++} generally being the most effective. Aronnson *et al.* (1978), after extensive purification of the human and pig red cell, rat liver and yeast enzymes, and atomic absorption analysis, said that all of them contained Zn^{++}. The mammalian enzymes have one Zn^{++} per subunit, or two per molecule. The fact that Mg^{++} restores activity to the apoenzyme more effectively than does Zn^{++} in most cases, if GLO is really a zinc metalloenzyme as indicated by the studies of Aronnson *et al.* (1978), seems to be a bit puzzling.

Schimandle and Vander Jagt (1979) purified red cell GLO from all three red cell phenotypes, obtaining 12,000 to 20,000 fold purifications. All the purified samples had MW 44,000, as estimated by gel filtration. The GLO from phenotypes 1 and 2 were indistinguishable kinetically, although the preparation from 2-1 red cells appeared to exhibit greater stability than that from either homozygous type.

Martin and Ott (1976) noted that substantial loss of GLO activity in hemolysates kept for several weeks can be slowed or prevented by the addition of bovine serum albumin at a concentration of 150 $\mu g/m\ell$ lysate. Racker (1951) had found that bovine albumin had a stabilizing influence on the yeast enzyme as well.

34.5 Medicolegal Applications

34.5.1 Disputed parentage

There are few papers as yet on the application of GLO to disputed parentage, because it is relatively new polymorphism. Because of the frequency of distribution of its phenotypes, however, GLO will undoubtedly turn out to be one of the better two allele systems available. Brinkmann and Püschel (1978) recommended the use of GLO typing in these cases, as did Eriksen (1979) who said that the exclusion probability was of the order of 18% in Denmark. The figure is very similar in Vienna (Pausch *et al.*, 1979), and in Poland (Jakliński and Koziol, 1979). There is little data on gene frequencies in U.S. populations as yet, but it does not seem unreasonable to expect that the probability of excluding a

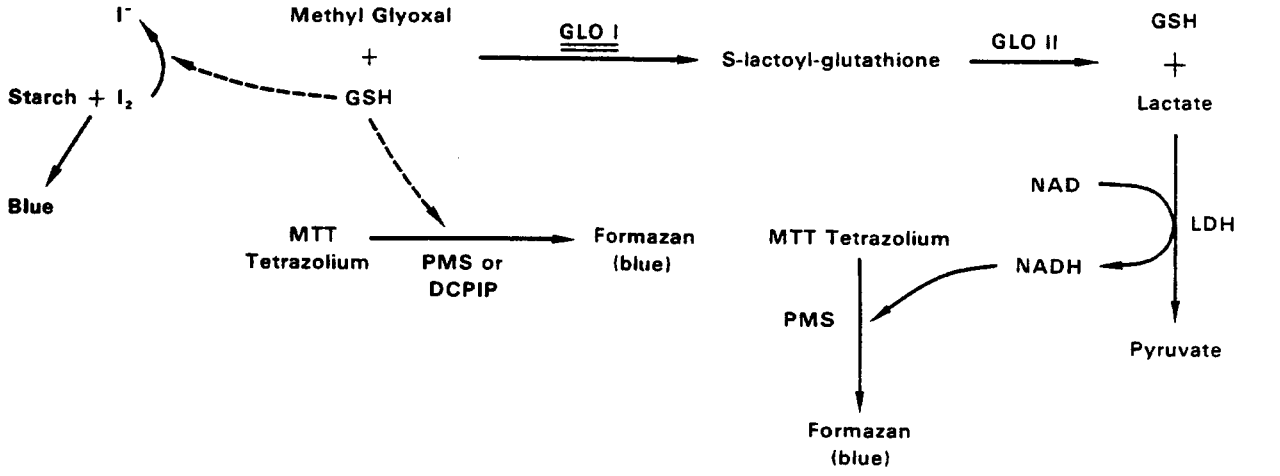


Figure 34.2 Detection Reaction Sequences for GLO

falsely accused father with the GLO system alone will be about 0.18 for Whites and about 0.16 for Blacks.

34.5.2 Electrophoretic methods for bloodstain typing

GLO has been phenotyped on starch gels and on several other electrophoretic media. Paar *et al.* (1977) used phosphate buffers at pH 6.7 for this system, while Kömpf *et al.* (1975) used a Tris-histidine buffer system at pH 7.8. The isoenzymes are quite acidic and both systems are perfectly suitable. Martin and Ott (1976) ran GLO isoenzymes on agarose gels in Tris buffers at pH 7.8. Migration is faster in agarose than in starch, and the plates could be run in less than 1½ hours. Meera Khan and Doppert (1976) gave a procedure for GLO typing on gelled cellulose acetate in pH 8 buffers.

Bloodstains can be phenotyped for GLO isoenzymes (Wraxall and Stolorow, 1978; MPFSL, 1978). The MPFSL utilized 0.2M phosphate buffers, pH 6.8, for the purpose. Stains are treated with dilute mercaptoethanol, and they said that mercaptoethanol and dithiothreitol were not interchangeable in this system. This procedure is given by Emes and Parkin (1980) as well.

Wraxall and Stolorow (1978) described a technique in which GLO phenotyping is combined with that of PGM and ESD in the same electrophoretic gel. The running gel is made from 2% starch and 1% agarose, and the original PGM buffer system of Spencer *et al.* (1964b) is employed. GLO isoenzymes run ahead of (anodal to) PGM and ESD isozymes in this system, and can be separately detected. Experience thus far indicates that GLO is quite labile in bloodstains, although detailed stability studies on bloodstains were not found in the literature. GLO is one of the better systems for bloodstain individualization. The DI for the White population is about 0.6, and that for the Black population will probably be a bit lower.

Burgess (1979) and Burgess and Twibell (1979) described a procedure for GLO phenotyping in hair root sheath cells using Cellogel membranes.

Burgess and Twibell (1979) said that Parkin had reported on GLO phenotyping in semen at the 7th International Congress of Forensic Haematogenetics in Hamburg in 1977 (I had no access to the proceedings of this conference). Blake and Sensabaugh (1978) reported that there is about 4 times more GLO in sperm cells than in red cells on a per cell basis, and that phenotypic expression in the two is identical. They said, however, that the GLO in seminal plasma showed different patterns which were difficult to interpret, and which tended to mask the clear patterns from the sperm cells. They suggested that GLO typing in whole semen would present very difficult problems. Stöhlmacher and Haferland (1980) recently showed that a variety of human tissues contained GLO activity, and that the same phenotype is seen in all the tissues (including red cells) from the same person. There was some variation in the concentration of GLO in the various tissues, which included skin, skeletal muscle, brain, tongue, tonsil, lung, heart, stomach, intestine, liver, spleen, kidney, various glands, testicle, uterus and bone marrow.

34.6 Distribution of GLO Phenotypes in U.S. Populations

Only two population studies were found before Table 34.1 was prepared. The table shows the data. Grunbaum *et al.* (1980) have added some further data for California Whites and Blacks, Mexicans from California and Mexico City and Asians from California and Hawaii. These frequencies were: For Whites (n=313): 54(17.3%) 1, 165 (52.7%) 2-1 and 94(30%) 2, GLO^1 = 0.436; For Blacks (n=308): 39(12.7%) 1, 125(40.6%) 2-1 and 146(47.4%) 2, GLO^1 = 0.327; For Mexicans (n=1080): 111(10.3%) 1, 444(41.1%) 2-1 and 525(48.6%) 2, GLO^1 = 0.308; For

Table 34.1 Distribution of GLO Phenotypes in U.S. Populations

Population	Total	Phenotype Distribution — Number (Percent) 1	2-1	2	*GLO*'★	Reference
CAUCASIAN						
Buffalo, NY	101	21 (20.7)	42 (41.6)	38 (37.6)	0.42	Weitkamp, 1976
Detroit, MI	503	100 (19.9)	260 (51.7)	143 (28.4)	0.457	Stolorow and Wraxall, 1978; Stolorow et al., 1979
NEGRO						
Buffalo, NY	107	10 (9.3)	40 (37.4)	58 (54.2)	0.28	Weitkamp, 1976
Detroit, MI	504	75 (14.9)	212 (42.1)	217 (43.1)	0.359	Stolorow and Wraxall, 1978; Stolorow et al., 1979

★ Gene frequency

Asians (n = 884): 4(0.5%) 1, 125(14.1%) 2-1 and 755 (85.4%) 2, GLO^1 = 0.075.

The gene frequency of GLO^1 in western European Caucasians is of the order of 0.4–0.5. It is lower in Negroes. GLO^1 is quite high in Japanese, about 0.9 (Toyomasu *et al.*, 1977), and apparently quite low in some Asians (Grunbaum *et al.*, 1980).

SECTION 35. GLUTAMIC-PYRUVIC TRANSAMINASE

35.1 Recognition of GPT

Glutamic-pyruvic transaminase (GPT; glutamate-pyruvate transaminase; glutamic-alanine transaminase; alanine aminotransferase; L-aspartate:2-oxoglutarate aminotransferase; E.C. 2.6.1.2) catalyzes the reversible conversion of L-alanine and 2-oxoglutaric acid to pyruvic acid and L-glutamic acid. 2-oxoglutaric acid was formerly called α-ketoglutaric acid. GPT is a representative of one category of amino group transferring enzymes, of which a number of categories are now known. There is a vast literature on transaminases. The review by Braunstein (1973) should be consulted for further details. "Glutamic-pyruvic transaminase" is an older name for this enzyme, but it is still commonly used by workers interested in genetic polymorphism because the designation "GPT" is the basis for the genetic variation nomenclature. Biochemists tend to call the enzyme "alanine aminotransferase", which is preferred by the Enzyme Commission as the trivial name.

Enzyme catalyzed aminotransferase activity involving amino acids and the corresponding 2-oxo acids was first described in 1937, and it was GPT activity that was detected (Braunstein and Kritzmann, 1937a, 1937b and 1937c). It was clear soon afterward that transaminase activities played a major role in amino acid metabolism. In 1944, Snell suggested that the vitamin B_6 related compounds pyridoxal phosphate and pyridoxamine might play a principal role as cofactors in these reactions, and his suggestion has turned out to be correct. Snell (1953) reviewed the development of knowledge about the role of pyridoxal phosphate in aminotransferase reactions. Green *et al.* (1945) presented evidence that pyridoxal phosphate was a required cofactor for pig heart GPT. GPT is widely distributed in nature. It has been known for quite some time that red cells possess GPT activity (Karmen *et al.*, 1955; Rapoport, 1961; Löhr and Waller, 1961; Radhakrishnamurty and Sabry, 1968). It is also known that GPT occurs in two forms in many animal tissues, one form being associated with mitochondria, and the other form being soluble (Hopper and Segal, 1964; Saier and Jenkins, 1967). GPT shares this property with GOT (E.C. 2.6.1.1). Biochemical studies on the aminotransferases have been periodically reviewed (Braunstein, 1947, 1957 and 1973; Meister, 1955). Many other reviews are cited by Braunstein (1973).

35.2 Genetic Variation of GPT

Genetic variation in GPT was discovered by Chen and Giblett in 1971. They detected three electrophoretic patterns in the bloods of different people after vertical starch gel electrophoresis of hemolysates in a Tris-citrate, pH 7.5, buffer system. The three phenotypes were designated "1", "2-1" and "2". Family studies indicated that a pair of autosomal codominant alleles was responsible for the phenotypes. These alleles were originally designated *Gpt*^{1} and *Gpt*^{2}, but are now more generally designated *GPT*^{1} and *GPT*^{2}. In a Seattle population, *GPT*^{1} showed a frequency of about 0.5 in Caucasians, 0.8 in Negroes and 0.6 in Orientals, making the system particularly powerful in discriminating members of a population. Family and population studies in a number of laboratories were consistent with the genetic explanation for the GPT polymorphism (Kömpf, 1971; Benkmann and Goedde, 1972; Chen *et al.*, 1972). An extensive study of the GPT phenotypes in a number of populations was carried out by Chen *et al.* (1972). Six additional rare phenotypes were observed in the course of the survey, which could be explained on the basis of heterozygosity of one of four new rare alleles with *GPT*^{1} or *GPT*^{2}. The new alleles were designated *GPT*^{3}, *GPT*^{4}, *GPT*^{5} and *GPT*^{6}. The *GPT*^{3} allele has been observed in a number of German populations (Brinkmann *et al.*, 1972c; Gussmann and Schwarzfischer, 1972; Martin and Niebuhr, 1973b; Jungwirth and Woll, 1974), and we found four examples of its expression in New York and London populations (Welch *et al.*, 1975). An additional allele has been described by Olaisen (1973a) segregating in four generations of a Norwegian family. This was called *GPT*^{7}, and examples of both GPT 7-1 and 7-2 were observed. The electrophoretic patterns shown by the different GPT phenotypes are indicated in Figure 35.1. A further allele, *GPT*^{8}, was observed by Santachiara Benerecetti *et al.* (1975) in an Italian population.

There are reports of silent alleles of GPT. Olaisen (1973b) found an apparent *GPT*^{0} segregating in a large family, and the presumed heterozygotes exhibited about half the GPT activity characteristic of the homozygous normal types. Spielmann *et al.* (1973) found another example in a survey of a German population. In this case, an apparent GPT 1 mother and GPT 2-1 father had a GPT 2 child, and the explanation appeared to be that this mother was *GPT*^{1}*GPT*^{0}, and her child, *GPT*^{2}*GPT*^{0}. The question of a silent allele at the *GPT* locus was called into question by the work of Kömpf and Bissbort (1974) and Kömpf *et al.* (1974). These investigators have presented evidence for inherited quantitative variability of the GPT isozyme activities. This matter is discussed more fully in section 35.4.2. Under some circumstances, typing results with certain quantitative 2-1 variants were almost indistinguishable from those that would be predicted for heterozygotes carrying a silent allele, unless densitometric traces were carefully examined. It does not appear that the issues surrounding *GPT*^{0} are fully settled as yet, and the subject is discussed further in section 35.5.1.

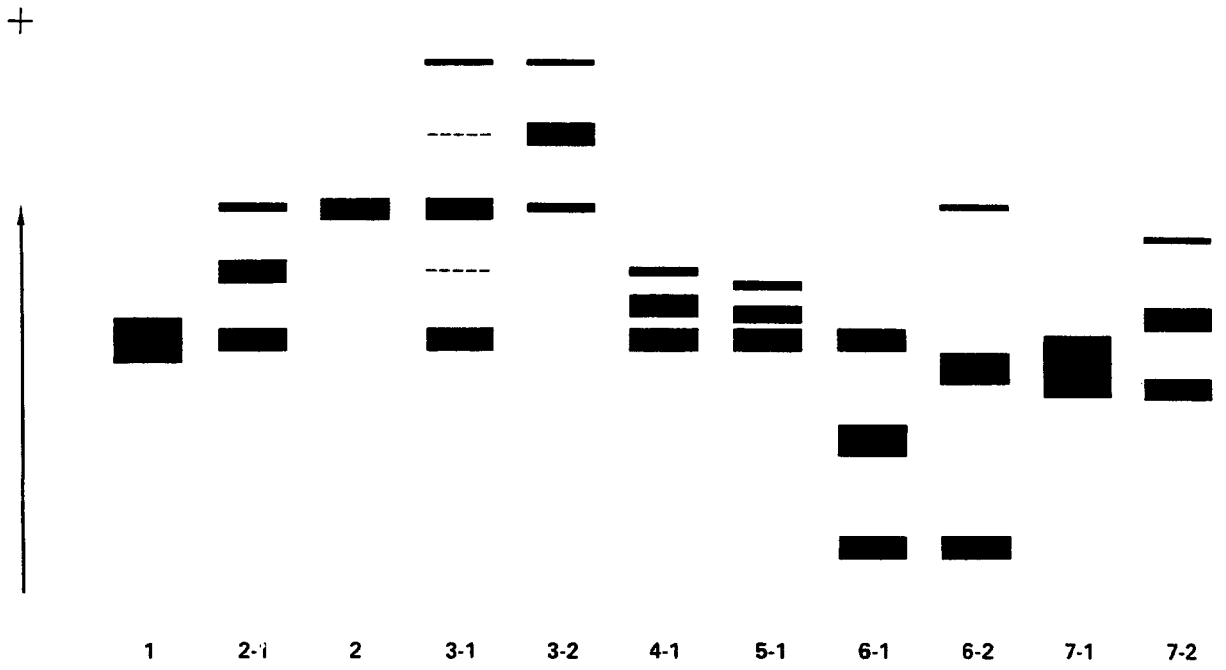


Figure 35.1 Electrophoretic Patterns of GPT Phenotypes

35.3 Procedures for GPT Phenotyping

The original studies which disclosed the GPT polymorphism (Chen and Giblett, 1971) employed vertical starch gel electrophoresis, but horizontal starch gel electrophoresis procedures have been successfully used as well (Radam and Strauch, 1972;. Welch, 1972a and 1972b; and others). Most workers use Tris-citrate buffers at pH 7.5 for electrophoresis, although systems have been described in which GPT is combined with other isoenzyme systems for typing in the same gel. GPT can be typed simultaneously with PGM, using the PGM buffer system (Gussmann and Rames, 1972), or with PGD, PGM and AK in Tris-citrate, pH 7.5, buffers (Goedde and Benkmann, 1972). Thick starch gels are normally employed for GPT typing, and they require slicing prior to staining. We have generally preferred to use thin starch gels, as described by Wraxall and Culliford (1968), for isoenzyme phenotyping, but this technique was not found to be very successful for GPT phenotyping. Part of the difficulty may have been that the red cell content of GPT is lower than that for many other polymorphic erythrocyte enzymes, and the thicker gel allows the application of more material for typing.

The detection of GPT in gels following electrophoresis has also been carried out in a number of different ways. Perhaps the most common is the original (Chen and Giblett, 1971) procedure (Figure 35.2, Sequence A). This method takes advantage of the fact that NADH fluoresces under the UV, whereas NAD does not, and zones of GPT activity show up under UV illumination as non-fluorescent bands on an otherwise fluorescent background. Methods in which MTT tetrazolium is added have been used as well. Since the zones of GPT activity are occupied by NAD, and since NADH is the reductant, these procedures result in clear bands on an otherwise purple-blue formazan background. Kömpf (1972) used this procedure. He incubated the gel with all the components except PMS, which was added in a second step. Anger *et al.* (1974) utilized a similar procedure except that they added all the components except MTT and PMS in the first overlay, incubated for about 2 1/2 hours, and then put on a second overlay containing MTT and PMS, after having removed the first one (Figure 35.2, Sequence A). Martin and Niebuhr (1973a) utilized a procedure based on a different reaction sequence. Alanine and 2-oxoglutarate were added as substrates, along with L-glutamic acid dehydrogenase, acetylpyridine adenine dinucleotide (APAD), MTT and PMS. The L-glutamate formed as the product of the GPT reaction is converted back to 2-oxoglutarate by L-glutamate dehydrogenase, with the concomitant reduction of APAD, which can reduce MTT to formazan. APAD is an NAD analog (Figure 35.2, Sequence B).

GPT typing can sometimes present problems (see, for example, Berg *et al.*, 1974a). The GPT^1 product is somewhat more active than that of GPT^2, and may stain more rapidly on the gels. The GPT 1 type may thus tend to become overstained if sufficient time is allowed for the full development of the GPT 2-1 and GPT 2 types. Overstained GPT 1 can

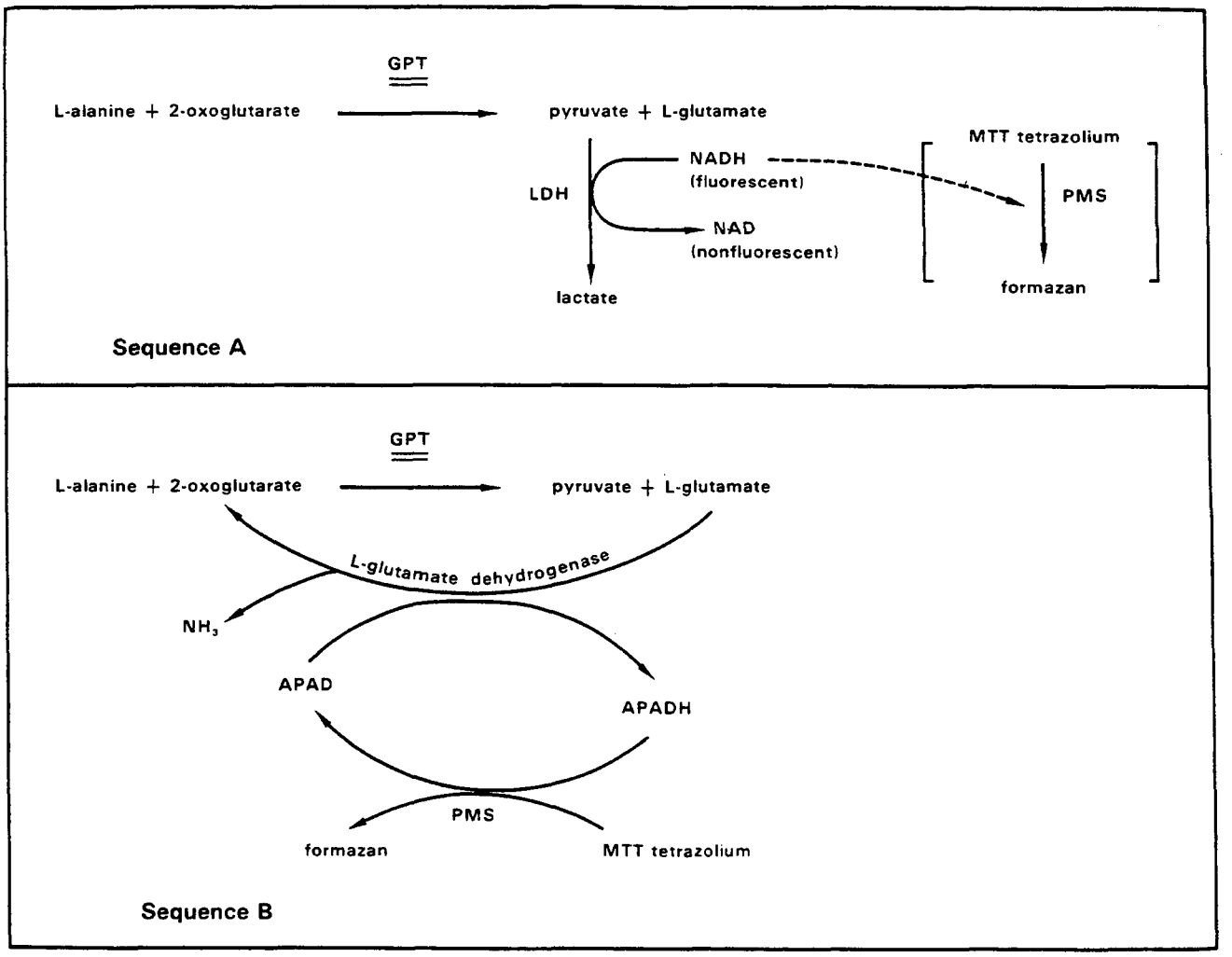


Figure 35.2 Detection Reaction Sequences for GPT

resemble GPT 2-1 at times. This difficulty can usually be avoided if the gels are examined several times during the course of development.

35.4 Biochemical Studies on GPT

35.4.1 Purification and properties of GPT

The biochemical studies on transaminase enzymes, including GPT, were reviewed by Braunstein in 1973. Transaminases, including GPT, require pyridoxal phosphate as a cofactor, and it is usually tightly bound to the enzyme. The cofactor is intimately involved in the catalytic mechanism, and Braunstain's (1973) review may be consulted for details.

The rat liver enzyme has been extensively purified, and crystallized (Gatehouse *et al.*, 1967; Matsuzawa and Segal, 1968). The molecule has a MW of 114,000 and contained 25 to 30 titrable -SH groups. There were two pyridoxal phosphate per molecule, and the enzyme exhibited a series of differently aggregated forms in the absence of mercaptoethanol. No reports of purification of the red cell enzyme were found.

35.4.2 Activity studies of the red cell GPT isozymes—quantitative variation

In 1972, Chen *et al.* carried out activity studies on the major GPT phenotypes. It was very clear that the *GPT*^{1} product had something more than twice the activity of the *GPT*^{2} product. The mean activity of heterozygote red cells fell in between the homozygous red cell values. Welch (1972a) conducted similar experiments, and his results also clearly showed that the *GPT*^{1} product was considerably more active than that of *GPT*^{2}. There was a fairly broad range of activity values within each phenotypic class, the 2-1 range overlapping both homozygous ranges. The 1-1 range did not overlap the 2-2 range, however. These activity differences were not based on any stability differences in the gene products that could be demonstrated by differential thermal

denaturation. The mean level of GPT 2-1 activity was in quite good agreement with the computed value from the mean activity levels of the homozygous phenotypes. Similar relationships between the activities of the isozymes were described by Ueda *et al.* (1979) using pyruvate and L-glutamate as substrates, i.e., measuring the "reverse" reaction.

In 1974, Kömpf *et al.* reported quantitative densitometric studies on the GPT phenotypes. In many cases, the three bands of the GPT 2-1 phenotype showed 4:3:2 activity ratios. But in a few cases, a different but consistent ratio was seen. These "variant" 2-1 types were seen not just in individuals, but in members of certain families. They were regarded, therefore, as genetic quantitative variants. One variant 2-1 exhibited an activity ratio of 1:1:1 in the three bands, and another variant showed 1:2:3. The latter was called a "2-1M" or "2-1 Marburg". The 2-1M, they said, would not be recognized as a 2-1 on an electrophoretic gel by visual reading, and they themselves had thought for some time that the pattern represented the product of a GPT^2GPT^0 combination. Quantitative assay data were collected by Kömpf and Bissbort (1974), following upon the densitometric work. Six discreet groups of activity level could be distinguished within the two homozygous phenotypic classes, they said, and four groups of activity level were present within GPT 2-1. They regarded these different levels of activity as being a manifestation of quantitative genetic variation of GPT (but see below in section 35.5.1).

35.5 Medicolegal Applications

35.5.1 Disputed parentage

GPT phenotypes are quite well distributed in many populations. The system is one of the better ones for population discrimination, and the GPT system is employed in a number of laboratories in disputed parentage investigations. Radam and Strauch (1972) noted that, in Berlin, 18.7% of falsely accused putative fathers would be excluded by GPT alone. The figure for England is very comparable at 19%, as noted by Welch and Dodd (1974), who reported their results in over 250 cases. The New York population data (Welch *et al.*, 1975) suggest that the value for American Caucasians would be 18 to 19%, while that for Blacks would be about 13%. The isozymes are fully developed in fetal blood (Chen *et al.*, 1977).

Although silent alleles are exceedingly rare, they can cause difficulty in the interpretation of disputed paternity results if encountered. Some of the material on GPT^0 was discussed in the foregoing sections, and there is some disagreement in the literature about interpreting the presence of a silent allele at the *GPT* locus. Heide *et al.* (1974) noted that the variation found in GPT activity within individual phenotypic classes was sufficiently great that enzymatic assays in individual samples could not be regarded as accurate in diagnosing the presence of a silent allele, especially in a medicolegal case. Their paper prompted Ritter to comment on the entire subject, based primarily on the work of Kömpf and others discussed in section 35.4.2 (Ritter, 1975a and 1975b). The comments were very technical, based upon the way the enzyme assays were carried out and their results interpreted. Heide *et al.* (1975) replied to Ritter's comments. They did not appear to accept the notion of genetically controlled quantitative variation in GPT phenotypic classes. The question of activity variation in GPT phenotypes, and the role of a silent allele in this variation, do not seem to have been completely settled as yet.

35.5.2 GPT phenotyping in bloodstains

GPT can be phenotyped in very fresh bloodstains. The system requires quite a bit more material for typing than many other systems, and more difficulty is encountered in trying to type GPT in bloodstains than in fresh blood. In 1972, Welch reported that GPT could be typed in bloodstains up to about 2 weeks old (Welch, 1972b). The electrophoretic system was very similar to that used for the typing of hemolysates. Pieces of bloodstained material about 15 mm^2 were briefly soaked, and inserted directly into the gel. Jungwirth and Woll (1974) also reported on GPT typing in bloodstains up to about a week old. Most laboratories do not carry out GPT typing in bloodstains, in part because only the freshest stains can be typed, and probably also in part because GPT is usually typed on thick starch gels. Most of the laboratories in this country use thin starch gels for enzyme typing, and it is difficult to put a sufficient amount of material into a thin gel for GPT typing. If suitable procedures could be devised for this enzyme, and if typing became possible in somewhat older stains, however, GPT would be one of the best enzymes for this kind of work because of its phenotypic distribution. The Seattle (Chen and Giblett, 1971; Chen *et al.*, 1972) and New York (Welch *et al.*, 1975) data suggest that the DI would be about 0.6 in Caucasians, and about 0.48 for Black Americans.

35.5.3 GPT in other tissues

GPT activity has been reported to occur in semen (Povda, 1962; Ishibe, 1975). It is not clear whether or not the "red cell" GPT types can be determined from seminal plasma or not. It has also been reported that GPT can be phenotyped in tooth parts (Suyama and Imai, 1975).

35.6 Distribution of GPT Phenotypes in U.S. Populations

Only two surveys were found for U.S. populations, and these data appear in Chen and Giblett (1971) and Chen *et al.* (1972) for Seattle, and in Welch *et al.* (1975) for New York.

SECTION 36. PEPTIDASES

36.1 Introduction to Peptidases

The peptidases found in human red cells and tissues are members of a diverse class of enzymes which catalyze the hydrolysis of peptide bonds. In the older literature, a distinction was made between endo- and exo-peptidases, the former being able to catalyze the hydrolysis of internal peptide bonds in peptide chains, while the latter catalyze the hydrolysis of terminal peptide linkages. Some of the endopeptidases show appreciable esterase activity, while many exopeptidases do not. The peptidases to be discussed in this section are exopeptidases, which can act on a variety of di- and tripeptides as substrates. They are also aminopeptidases, i.e., hydrolyzing the N-terminal peptide bond, rather than carboxypeptidases which hydrolyze C-terminal peptide linkages. The earlier literature on peptide bond cleavage was reviewed by Smith (1960), and the more recent review by Delange and Smith (1971) contains some information on the types of peptidases which are discussed in this section.

Peptidases A, B, C, E, F and S are also called dipeptidases, tripeptidases, and aminopeptidases, and are classified by the Enzyme Commission under E.C. 3.4.11.X and 3.4.13.X, where the "X" can be one of several different numbers. Peptidase D is also called proline dipeptidase, prolidase and imidodipeptidase, and is classified under E.C. 3.4.13.9.

36.2 Peptidases of Human Red Cells and Tissues—Multiple Genetic Loci Determining Peptidases

In 1952, Adams *et al.* showed that four distinct di- and tripeptidases were present in human red cells. The substrates used for the different enzymes included gly-pro (for prolidase), L-leucinamide (for aminopeptidase), and di- and triglycine. Haschen (1961) characterized the gly-leu peptidase activity of red cells in terms of its kinetics and activation mechanism, and showed that the enzyme requires Zn^{++} as an essential cofactor. In 1963, Haschen studied the kinetic properties of human red cell prolinase and prolidase.

In 1967, Lewis and Harris subjected red cell hemolysates to starch gel electrophoresis, and stained the gels for peptidase activity with a series of di- and tripeptides as substrates. Based upon electrophoretic mobility and substrate specificity, five distinct types of peptidase could be identified in human red cells, and these were designated A, B, C, D and E. Peptidases A and C showed a broad range of substrate specificity, but did not hydrolyze any of the tripeptides tested (leu-gly-gly, leu-gly-phe, tyr-tyr-tyr and leu-leu-leu). Peptidase B was the only one that hydrolyzed the tripeptides (it was most active with leu-gly-gly), and it also hydrolyzed phe-leu, phe-tyr, pro-phe and lys-leu. Peptidase B may correspond to the "tripeptidase" described by Adams *et al.* (1952), which was detected with triglycine. Peptidase D reacted only with leu-pro, of the substrates tested, and corresponds in all probability to the "prolidase" of Adams *et al.* (1952) and Haschen (1963). Prolidases are peptidases that catalyze the hydrolysis of various peptides having proline as C-terminus. Peptidase E reacted with dileucine, lys-leu, phe-leu and phe-tyr, but its activity was relatively weak. It was the only one of the peptidases that hydrolyzed leucyl-β-naphthylamide. The list of substrates tested with the various peptidases has since been expanded (see Table 36.1). The peptidases are designated "PEP", followed by the appropriate letter, i.e., PEPA, PEPB, etc.

The designations "A" through "E" were based partly on electrophoretic mobility, A being the slowest and E being the fastest (most anodal) in the system used in these experiments (Figure 36.1). Electrophoresis was carried out on starch gels, with bridge buffer consisting of 0.1M Tris-maleic acid, pH 7.4, and gel buffer consisting of 1:20 dilute bridge buffer at the same pH. Detection of peptidase activity following electrophoresis was carried out by allowing the L-amino acids produced in the reaction to react with oxygen via the L-amino acid oxidase reaction, and detecting the peroxide thus formed with o-dianisidine in the presence of peroxidase (Figure 36.2 A). In 1969, Harris reported a zone of peptidase activity which had an electrophoretic mobility more anodal than E, and it was called PEP F. It showed very weak activity compared to the other peptidases, and utilized only trileucine and trityrosine as substrates.

In 1971, Rapley *et al.* carried out an extensive study of tissue distributions of various peptidases. The peptidases were found to be distributed in most tissues examined, although different tissues showed differing amounts of the various peptidase isozymes in most cases. A further peptidase activity, designated PEP S, was found in all tissues examined, except red cells, skin and saliva. Its electrophoretic mobility is slower than (cathodal to) PEP A in the pH 7.4 buffer system of Lewis and Harris (1967). PEP S showed a broad range of substrate specificity, and was especially active with dileucine, phe-leu and trileucine.

The data collected in these studies, coupled with the biochemical studies (section 36.5) and the observation of genetic variation in a number of the different peptidases (section 36.4), have led to the conclusion that the different peptidases are under the control of different genetic loci, and all subsequent experimental data is consistent with this data. The genetic loci are designated *PEPA, PEPB, PEPC,* and so forth.

The relative electrophoretic mobilities of peptidases A through F and S are shown diagramatically in Figure 36.1.

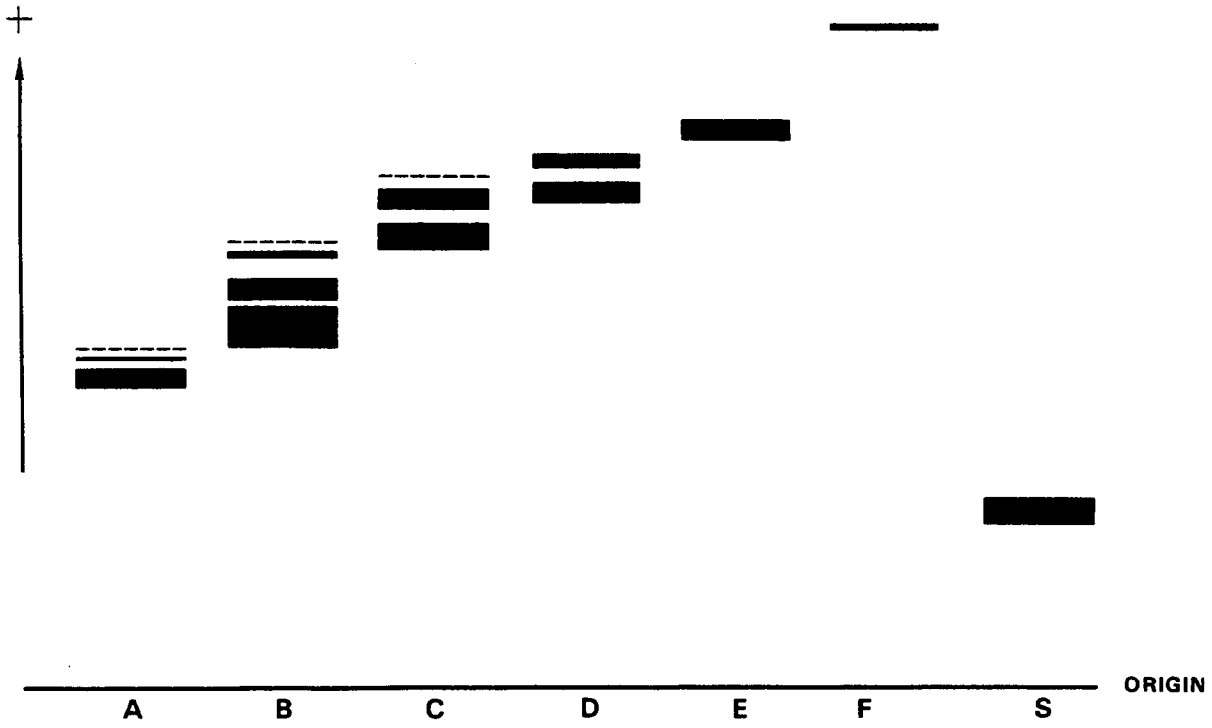


Figure 36.1 Relative Electrophoretic Mobilities of Peptidases A through F and S (after Harris and Hopkinson, 1976)

The activities of the various peptidase isoenzymes toward a variety of substrates is shown in Table 36.1. This information may also be found in Harris and Hopkinson (1976).

36.3 Detection of Peptidase Activity

Lewis and Harris (1967) detected peptidase activity through the use of a coupled reaction involving the L-amino acid oxidase reaction. The scheme is shown in Figure 36.2 A. In this sequence, it is the peroxide resulting from the L-amino acid oxidase reaction that is actually detected by the addition of oxidizable dye (o-dianisidine) and peroxidase. The L-amino acid oxidase normally available comes from snake venom, and this enzyme is only reactive with leucine, isoleucine, methionine, phenylalanine, tyrosine or tryptophan to any significant extent. This feature of the detection scheme, therefore, restricts the di- and tripeptides which can be used as substrates for the peptidases. Substrates must be selected which will produce one of the amino acids as products which can, in turn, react with the snake venom L-amino acid oxidase. Dyes other than o-dianisidine have also been employed. For spectrophotometric assay of the enzyme, Sinha *et al.* (1970) used the same scheme that is represented in Figure 36.2 A, but the dye was o-tolidine. The oxidized o-tolidine product absorbs visible light at 415 nm. Baker (1974) introduced another dye for the staining procedure, called 3-amino-9-ethyl carbazole. This substance is oxidizable to a brown-colored product. Harris and Hopkinson (1976) recommended its use, rather than o-tolidine or o-dianisidine.

In 1971, Rapley *et al.*, in connection with their extensive study of the tissue peptidases, introduced a second detection system based upon the GPT reaction (compare Figure 35.2, Sequence A). GPT reacts with 2-oxoglutarate and alanine, and this detection scheme, which is shown in Figure 36.2 B, allows another whole range of di- and tripeptides which release alanine to be used as substrates for the peptidase isoenzymes.

For routine work, Hopkinson and Harris (1976) recommended the use of the following substrates for the peptidase isozymes: leu-ala for PEP A and PEP C; leu-gly-gly for PEP B; leu-pro or phe-pro for PEP D; and trileucine for PEP S, B, E and F. It should be noted that the peptidases are specific for peptides made from L-amino acids. Glycine, of course, is not optically active.

Kühnl *et al.* (1979) noted that PEP activity with glycine-containing peptides can be detected by allowing the glycine produced to react with o-phthalaldehyde. This method was used to detect PEP A activity following electrohoresis.

Table 36.1 Activities of Various Peptidases with a Number of Substrates (after Rapley et al., 1971)

	Activity of Peptidase						
Substrate	**A**	**B**	**C**	**D**	**E**	**F**	**S**
Val-Leu	+++	−	−	−	−	−	++
Val-Ala	+++	−	−	−	−	−	+
Ala-Gly	+++	−	−	−	−	−	++
Gly-Leu	+++	−	+	−	−	−	−
Leu-Gly	+++	−	+	−	−	−	+
Gly-Phe	++	−	+	−	−	−	−
Ala-Lys	++	−	+	−	−	−	+
Ala-Glu	++	−	+	−	−	−	−
Leu-Ala	++	−	+++	−	−	−	++
Gly-Ala	++	−	++	−	−	−	−
Leu-Leu	++	−	++	−	+	−	+++
Gly-Trp	++	−	++	−	−	−	+
Ala-Ala	+	−	±	−	−	−	−
Lys-Leu	+	+	+++	−	+	−	++
Lys-Tyr	+	+	+++	−	+	−	++
Pro-Phe	++	+	+++	−	−	−	±
Pro-Leu	++	+	+++	−	−	−	−
Phe-Leu	++	++	+++	−	+	−	+++
Phe-Tyr	++	++	+++	−	+	−	+++
Leu-Tyr	+++	++	+++	−	+	−	+++
Ala-Tyr	++	+++	+	−	±	−	+
Ala-His	++	−	++	−	−	−	+
Leu-Gly-Gly	−	+++	−	−	±	−	+
Ala-Gly-Gly	−	+++	−	−	−	−	++
Ala-Ala-Ala	−	+++	−	−	+	−	++
Leu-Leu-Leu	−	++	−	−	+	+	+++
Leu-Gly-Phe	−	+	−	−	±	−	+
Tyr-Tyr-Tyr	−	+	−	−	±	+	+
Phe-Gly-Phe-Gly	−	−	−	−	++	−	+
Leu-Pro	−	−	−	++	−	−	−
Phe-Pro	−	−	−	++	−	−	−
Ala-Pro	−	−	−	++	−	−	−
Leu-β-Naphthylamide	−	−	−	−	+	−	+
Leucinamide	−	−	−	−	±	−	+
Leu-Nitroanilide	−	−	−	−	+	−	±

36.4 Genetic Variation of the Peptidases

36.4.1 PEP A

Lewis and Harris (1967) in the original paper on red cell peptidases found five PEP A patterns in the course of examining a larger number of hemolysates. These were designated PEP A 1, 2-1, 2, 3-1 and 4-1, and family studies indicated that they could be explained by four alleles, *PEPA*^{1}, *PEPA*^{2}, *PEPA*^{3} and *PEPA*^{4}, at an autosomal locus. *PEPA*^{3} and *PEPA*^{4} are very rare. *PEPA*^{2} is relatively frequent in Black populations, but is exceedingly rare in Europeans. Some 15%–20% of Blacks in many African populations are PEP A 2-1. In 1968, two additional rare alleles of PEP A, *PEPA*^{5} and *PEPA*^{6}, were described by Lewis *et al.*. Both were seen as heterozygotes, PEP A 5-1 and 6-1. In 1969, Harris described another rare allele, *PEPA*^{7}, which had been found as a 7-1. In 1973, Lewis added *PEPA*^{8} to the list. The *PEPA*^{8} product exhibits very low activity in red cells, but can be detected in leucocytes, placentae and fibroblasts as well as in other tissues. *PEPA*^{8} occurs at polymorphic frequency (about 0.25) in Europeans, but its frequency is much lower in Nigerians (about 0.08). The *PEPA*^{8} phenotypes are discussed further in what follows in connection with the quantitative variation of PEP A in red cells.

In 1970, Sinha *et al.* undertook studies of the activity of PEP A in the red cells of a number of people. The common PEP A 1 types showed a considerable variation in activity levels, which was much less pronounced in white cells. Parent-child studies of this variation suggested that the activity levels were under genetic control, and Sinha *et al.* (1970) proposed that there might be two *PEPA*^{1} alleles, which they called *PEPA*^{1S} and *PEPA*^{1W} (where the "S" and "W" indicated "strong" and "weak"). In 1971, Sinha *et al.* carried out follow-up studies on the activity variation. PEP A isozymes from the red cells of persons with "strong" and "weak" activity were partially purified, and their properties compared in the hope of finding evidence of qualitative differences in the enzymes. Both types of enzyme, however, were completely similar with respect to pH-activity profile, K_m, elution profiles from ion exchange media and thermostability. In 1973, Lewis noted in his description of *PEPA*^{8} that the products of *PEPA*^{1} and *PEPA*^{8} cannot be separated adequately by the usual electrophoretic system. He used 0.3M potassium phosphate, pH 5.9, bridge buffer with an 0.02M imidazole-citrate, pH 5.9, gel buffer system on 11% starch gels to distinguish the PEP A 8-1 and 8 phenotypes from PEP A 1. The finding of very low activity of the *PEPA*^{8} product in red cells, the fact that *PEPA*^{1} and *PEPA*^{8} products were not separated by the usual electrophoretic

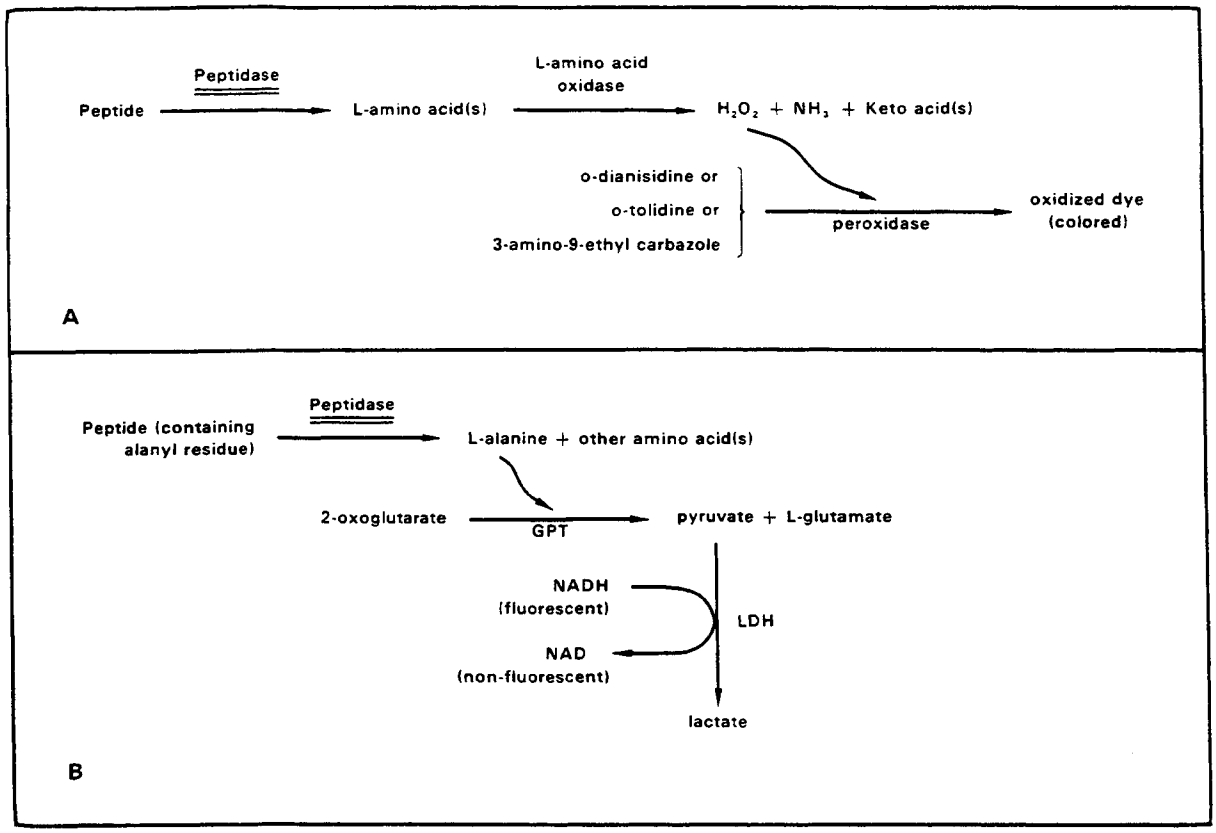


Figure 36.2 Schemes for the Detection of Peptidase Activity

system, and the finding that PEP A 8–1 occurred in appreciable frequencies in Europeans, led him to suggest that the *PEPA*^{1S} of Sinha *et al.* (1970 and 1971) was in fact *PEPA*^{1}, while the "*PEPA*^{1W}" was, in reality, *PEPA*^{8}. This notion appears to be the currently accepted one (Harris and Hopkinson, 1976). *PEPA*^{8} phenotypes are difficult to detect in red cells, because of the low activity of the *PEPA*^{8} product, and it is necessary to use a different electrophoretic system, as described by Lewis (1973), in order to get clear separations. An additional allele of PEP A, called *PEPA*^{9}, which gives rise to an unstable isoenzyme product *in vivo*, has been described by Kühnl *et al.* (1979) in a German family.

36.4.2 PEP B

All the variants of PEP B are very rare. Lewis and Harris (1967) detected four phenotypes of PEP B, and called them 1, 2–1, 3–1 and 4–1. Family studies indicated that they were the expression of four alleles at an autosomal locus, *PEPB*^{1}, *PEPB*^{2}, *PEPB*^{3} and *PEPB*^{4}. Only 9 Europeans in a survey of 2,197 people showed a phenotype other than PEP B 1. In 1970, Blake *et al.* reported four additional types, explicable on the basis of two additional rare alleles, *PEPB*^{5} and *PEPB*^{6}. Examples of PEP B 6–1, 7–1 and an apparent homozygous PEP B 6, were seen in Australian aborigines, and a PEP B 5–1 was detected in a survey of Nigerians.

36.4.3 PEP C

In 1970, Santachiara Benerecetti found genetic variation in PEP C in the Babinga pygmies of Africa. Two additional phenotypes, besides the common one, which were called 2–1 and 2, could be explained by a second allele at the *PEPC* locus, *PEPC*^{2}. The frequency of this allele was fairly low (0.014). In addition, 11 of 261 people had no detectable PEP C activity in hemolysates, and this was attributable to homozygosity for a silent allele, *PEPC*^{0}, which had a frequency in these people of about 0.21.

In 1972, Povey *et al.* described five additional phenotypes of PEP C, called 3–1, 4–1, 3, 4 and 5–1. These were accounted for by the additional alleles *PEPC*^{3}, *PEPC*^{4} and *PEPC*^{5}. *PEPC*^{5} is very rare, and *PEPC*^{3} and *PEPC*^{4} occur in Europeans at frequencies of less than 0.01. The *PEPC*^{4} product has the same electrophoretic mobility as the *PEPC*^{3} one. The *PEPC*^{4} isozyme is unstable in red cells, presumably because it is labile in the anucleate phase of red cell life, and it is almost inactive in hemolysates. In red cells, therefore, PEP C 4–1 and 1–1 are not distinguishable, and white cells or tissues must be examined for this purpose. *PEPC*^{4} had a frequency of about 0.08 in the Black people examined by Povey *et al.* (1972). *PEPC*^{4} may be identical to the "*PEPC*^{0}" of Santachiara Benercetti (1970), since the latter investigator examined only red cells.

36.4.4 PEP D

Lewis and Harris (1969a) reported three phenotypes of PEP D in addition to the usual PEP D 1. These were called PEP D 2–1, 2 and 3–1, and represented the expression of two additional alleles, *PEPD*^{2} and *PEPD*^{3}. Both of these alleles are quite rare, but have frequencies of about 0.024 and 0.021 in African Black populations (Harris and Hopkinson, 1976).

A rare disorder, apparently inherited as a recessive trait, and characterized by the absence of red cell PEP D activity, was reported by Powell *et al.* (1974). There were a considerable number of peptides (15 were found in a 24 hour sample) excreted in the urine of the subject, and all were either di- or tripeptides with C-terminal proline.

36.4.5 Linkage relations of the *PEP* loci

Family investigations by Lewis and Harris (1967) suggested that the *PEPA* and *PEPB* loci were not closely linked. It has since been shown by means of human-rodent somatic cell hybrid studies that *PEPA* is on chromosome 18 (Creagan *et al.*, 1973a), *PEPB* is on chromosome 12 (Chen *et al.*, 1973), and *PEPC* is syntenic to *PGM*$_1$ and *Rh* on chromosome 1 (Ruddle *et al.*, 1972).

36.5 Biochemical Studies on the Peptidases

The electrophoretic patterns observed with PEP A 1, 2–1 and 2 phenotypes suggested to Lewis and Harris (1967) that PEP A was a dimeric molecule, and Lewis and Harris (1969c) carried out molecular hybridization experiments with PEP A from different phenotypes to establish that this was indeed the case. The product of the rare *PEPA*^{5} allele is affected by sulfhydryl reagents in a manner suggestive of a free -SH group in the protein (Lewis *et al.*, 1968; Sinha and Hopkinson, 1969). The enzyme undergoes changes upon storage which can be mimicked by treatment with oxidized glutathione (GSSG), and which are reversed by treatment with mercaptoethanol. The electrophoretic patterns of PEP B and PEP C variants suggest that these are monomeric isozymes, while those of PEP D indicate that it, like PEP A, is dimeric.

The MW of all the peptidases have been estimated by gel filtration chromatography (Lewis and Harris, 1969b; Rapley *et al.*, 1971; Harris, 1969): PEP A, 92,000; PEP B, 55,000; PEP C, 64,400; PEP D, 100,000; and PEP S, 245,000. PEP E from all tissues except liver has a MW of 85,000, but the liver PEP E is about 177,000.

36.6 Medicolegal Applications

No reports on the application of the peptidase polymorphisms to disputed parentage investigations were found. Chakraborty *et al.* (1974) said that their calculations indicated an exclusion probability of about 7% for falsely accused Black men with the PEP A system. The PEP isozymes are fully developed in fetal blood (Chen *et al.*, 1977).

The PEP A polymorphism has been applied to bloodstain grouping, but it is not widely used. PEP A is not significantly polymorphic in Caucasians, but the *PEPA*^{2} allele occurs at polymorphic frequency in many Black populations. Culliford (1971) noted that PEP A phenotypes could be determined in bloodstains, but that the system taken alone was not very economical, because of the expense of the reagents and the low frequency of variants. The system is

apparently in routine use, at least in selected cases, in London at the present time, however (MPFSL, 1978). PEP A phenotyping is carried out on 10% thin starch gels in 0.1M Tris-phosphate bridge buffer, pH 7.4, and 0.01M Tris-maleate gel buffer, pH 7.5. This procedure is also described by Parkin (1978). The enzyme is detected using the scheme shown in Figure 36.2 A, with aminoethylcarbazole.

In 1976, Neilson *et al.* described an electrophoretic procedure in which PEP A phenotyping was combined with that of PGM and AK on the same electrophoretic gel. The original PGM buffers, described by Spencer *et al.* (1964b) were employed, with 10% thin starch gels, except that the EDTA was omitted in the bridge buffer, and combined instead with the PGM reaction buffer, and the bridge buffer was diluted 1:10 to make gel buffer. The procedure was applicable to bloodstain typing.

The data of Blake (1976) and Blake and Sensabaugh (1976) clearly show that PEP A is expressed in seminal plasma and in spermatozoa. The majority of the activity occurs in the seminal plasma rather than in the cells, and PEP A is unusual in that semen contains considerably more of it than a comparable volume of blood (some 25 times more) (Blake and Sensabaugh, 1978). Accordingly, quantity is not a limiting factor in seminal PEP A typing. The only variant type that is comparatively well distributed and can be detected relatively easily by electrophoresis, however, is largely limited to Black populations. Development of a reliable procedure for the detection of $PEPA^{3}$ (or $PEPA^{1W}$) would make the system a useful marker in White populations as well. Parkin (1979) said that PEP A can be typed in semen, but that vaginal swabs containing semen are typable only if they were taken within a few hours after intercourse.

Neilson *et al.* (1976) reported that 157 Black Americans, from the greater Pittsburgh area, had been phenotyped for PEP A with the following results: 139(88.5%) 1, 13(8.3%) 2-1, 4(2.5%) 2 and 1(0.6%) 3-1. The gene frequencies for this population would thus be $PEPA^{1} = 0.93$ and $PEPA^{2} = 0.07$. Grunbaum *et al.* (1980) tested 301 Whites and 492 Blacks from California, 766 Mexicans from California and Mexico, and 108 Asians from California and Hawaii. 300 of the Whites were PEPA 1 (1 was rare) and all but 5 of the Mexicans were PEPA 1 (3 were 2-1; 2 were rare). The Asians were all PEPA 1. Among the Blacks were 442(89.8%) 1, 41 (8.3%) 2-1, 5(1%) 2 and 4(0.8%) rare, and $PEPA^{1} = 0.948$, $PEPA^{2} = 0.052$. The patterns of the commoner PEP A types seen in Black populations are shown in Figure 36.3.

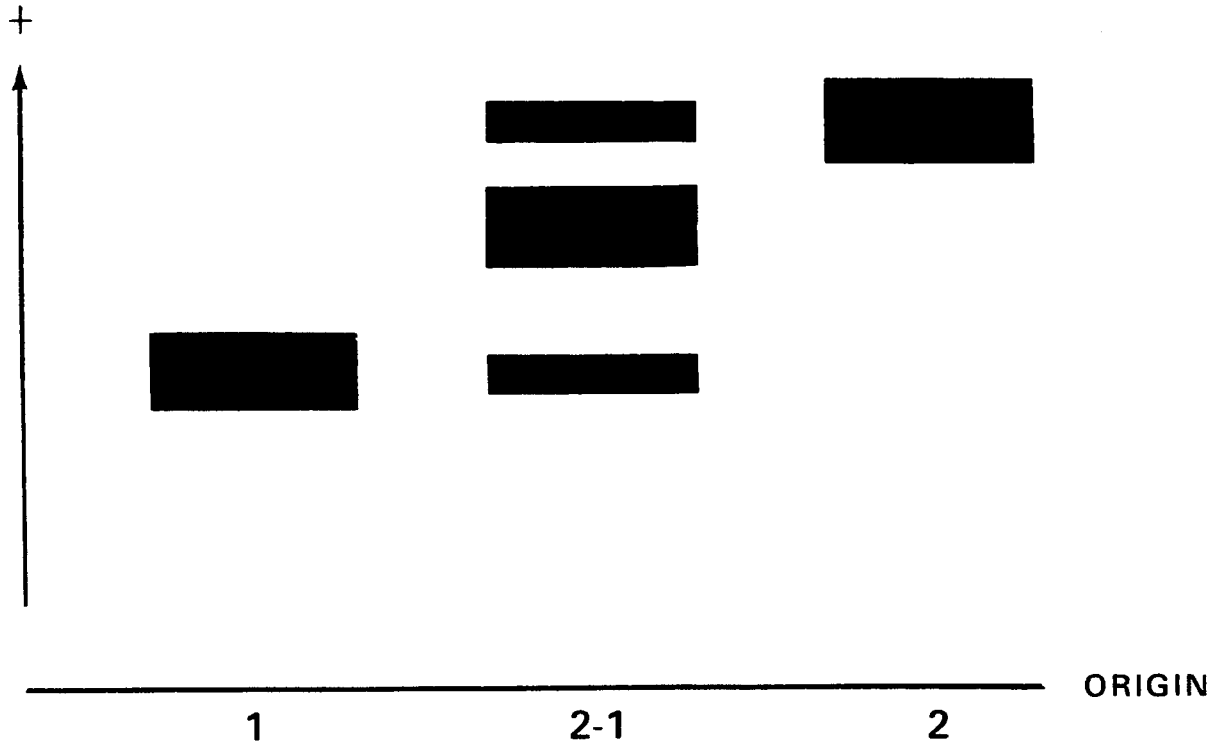


Figure 36.3 Electrophoretic Patterns of *PEPA* Phenotypes in Black Populations

SECTION 37. OTHER ISOENZYME SYSTEMS

37.1 Introduction

There are a considerable number of human enzymes, besides those discussed in sections 27 through 36, which exhibit genetically determined multiple molecular forms. A number of these are not expressed either in blood or in body fluids of the kinds encountered in criminal investigations, nor in tissues or fluids which would be accessible in disputed parentage cases. Accordingly, they are not useful genetic markers from a medicolegal point of view. Some of these isozymes are expressed in blood, however. In certain cases, procedures for their routine use in medicolegal cases have not been worked out. In other cases, the genetic variation does not occur with a high enough frequency in populations to make them worth the investment of time and material required for their phenotyping. A number of these additional systems are discussed briefly in this section.

37.2 Alkaline Phosphatase [ALP; orthophosphoric-monoester phosphohydrolase (alkaline optimum); E.C. 3.1.3.1]

37.2.1 Alkaline phosphatases of serum and tissues

A number of different alkaline phosphatases have been described in human serum and tissues. These enzymes have been of interest to clinical chemists and biochemists for years. There is a very large literature on the subject, which will not be developed in detail here. There are a number of excellent reviews which may be consulted for details. The material up to 1959 was reviewed by Gutman (1959), one of the pioneers in the field. More recently, Posen (1967), Fishman and Ghosh (1967) and Fishman (1974) have reviewed the subject.

Several different organs are now known to contribute to the alkaline phosphatase activity of serum, notably bone, liver, intestine and placenta. These different isoenzymes are distinguishable from one another on the basis of a combination of properties, including electrophoretic mobility, inhibition by specific inhibitors such as L-phenylalanine and L-homoarginine, immunological cross reactivity, and the effect of treatment with neuraminidase. All the evidence is consistent with these forms being under the control of different genetic loci. Following Harris and Hopkinson (1976), the liver, bone and intestinal enzymes are designated ALP_L, ALP_B and ALP_I, respectively. These isozymes have not been reported to exhibit genetic variation. From a medicolegal standpoint, therefore, they do not serve as genetic markers. There has been reported a rare familial type of low serum ALP activity (hypophosphatasia), which may be accompanied by widely varying degrees of skeletal abnormalities in affected individuals. The condition is inherited as an autosomal recessive, and is also characterized by the excretion of phosphoethanolamine in urine. It is apparently the skeletal, or bone, isoenzyme (ALP_B) that is deficient in this syndrome, because levels of intestinal enzyme (ALP_I) are normal (Rathbun *et al.*, 1961; Danovitch *et al.*, 1968; Warshaw *et al.*, 1971).

Efforts have been made to utilize the concentration of alkaline phosphatase in body fluids as a tissue marker, especially in the case of saliva. There is no doubt that saliva exhibits ALP activity (Giri, 1936; Chauncey *et al.*, 1954; Levitskii *et al.*, 1973; Lindqvist *et al.*, 1974; Pini Prato, 1970). This matter has been discussed in section 11.2. The placental alkaline phosphatase has been shown to exhibit genetic polymorphism (section 37.2.2). This enzyme is designated "PL" (Harris and Hopkinson, 1976). The placental enzyme is responsible for an elevation of serum ALP activity during pregnancy (Fishman *et al.*, 1972), and this fact has been used as the basis of medicolegal identification tests for the diagnosis of blood from pregnant women (Oya *et al.*, 1973; Stafunsky and Oepen, 1977; and see in section 8.2.4).

37.2.2 Placental alkaline phosphatase (PL)

In 1961, Boyer observed several different electrophoretically distinguishable phenotypes of the alkaline phosphatase from placentae. In 1965, Robson and Harris established that there were six relatively common phenotypes, distinguishable by electrophoresis provided the sample were run at both pH 8.6 and pH 6. At either of these pH values alone, certain of the phenotypes are indistinguishable from one another. The phenotypes were originaly called S, FS, SI, I, FI and F, and the designations referred to "slow", "intermediate" and "fast" electrophoretic mobilities. In 1967, further variants were discovered by Robson and Harris, and the nomenclature was revised to include numerical subscripts to distinguish between certain types, e.g. F_1S_1 and F_1S_2. An extensive study by Donald and Robson (1974) revealed a large number of rare variants. They described 44 phenotypes in all, and these could be accounted for by a series of 18 separate alleles at the *PL* locus, all except three of which are rare in all populations studied. The nomenclature was revised by Donald and Robson (1974) to a numerical system, the three common alleles being designated PL^1, PL^2 and PL^3. The common phenotypes, PL 1, 2-1, 2, 3-1, 3-2 and 3 correspond to the older designations S_1, F_1S_1, F_1, S_1I_1, F_1I_1 and I_1, and to the still older designations S, FS, F, SI, FI and I, respectively. The remainder of the *PL* alleles have been designated PL^4 through PL^{18}. The expression of placental alkaline phosphatase is controlled by the genotype of the fetus, and not of the mother. It has been

suggested (Gladkikh, 1976) that this interesting polymorphism might find medicolegal applications in certain types of cases.

37.2.3 Other notes on alkaline phosphatases

The placental enzyme has been studied by many workers, including Beckman and Beckman (1968), Beckman (1970), Posen *et al.* (1969) and Beratis and Hirschhorn (1972). The enzyme has also been purified and characterized in comparison with the ALP from other tissues (Ghosh, 1969). There is an established relationship between the level of intestinal ALP and the ABO type and secretor status of individuals (Beckman and Beckman, 1970). This enzyme is rarely found in the serum of nonsecretors, and among secretors, the highest levels are seen in persons of group A and O. There is no such relationship between ABO group, secretor status and the placental enzyme. The function of the alkaline phosphatases *in vivo* is not really known. The ALP isozymes from the different tissues may become elevated in serum in certain disease states involving the tissue of origin, and this has been a subject of great clinical interest because of the obvious diagnostic implications. There has been great interest, too, in the finding that a placental-like ALP isozyme occurred in the serum of a male cancer patient. This isoenzyme has been called the "Regan isoenzyme" after its first known possessor (Fishman *et al.*, 1968a and 1968b; Fishman, 1969). Other ALP isozymes resembling, or having properties in common with, placental alkaline phosphatase have been found in the sera of other cancer patients as well. Fishman (1974) regarded the issue of the identity of the placental isozyme and that which occurs in the serum of cancer patients as settled, and, in fact, uses the term "carcinoplacental isoenzyme antigen" to describe them because of their immunological identity. Beckman (1978), however, did not seem to regard the identity of the Regan isoenzyme and placental alkaline phosphatase as proven.

37.3 α-Amylase [AMY; diastase; ptyalin; 1,4-α-D-glucan glucanohydrolase; E.C. 3.2.1.1]

37.3.1 Activity and occurrence of α-Amylase

α-Amylase catalyzes the endohydrolysis of 1→4 α-glycosidic linkages in starch, glycogen and related polysaccharides containing three or more sugar residues, in a random manner. Amylase is one of the oldest known enzymes, its activity in pancreatic extracts having been noted and measured by Roberts in 1881. Amylases are primarily of two types, pancreatic and salivary, and the amylase activity of serum and urine is believed to be primarily pancreatic in origin. Skude (1977) gave a thorough discussion of the amylases in human serum. The isoenzymes which occur in serum can give clinical indications (Warshaw, 1977). Salivary amylase is the basis of several identification tests for the presence of saliva (see section 11.3).

37.3.2 Genetics of salivary and pancreatic amylases (AMY_1 and AMY_2)

It has been known for some time that both salivary and pancreatic amylases exhibit multiple molecular forms (McGeachin and Reynolds, 1961; Ogita, 1966; McGeachin, 1968). In 1965, Kamarýt and Laxova reported observations on an unusual band pattern in the serum amylases, and studies on the family indicated that the pattern was inherited. Further variants, which appeared to be segregating in families, were reported the following year (Kamarýt and Laxova, 1966). These workers used agar gel electrophoresis for the separaton of the isoenzymes, and the serum patterns were believed to be the result of both salivary and pancreatic contributions to amylase activity. Starch gels cannot be used for amylase electrophoresis for obvious reasons, and most workers have employed either agar or polyacrylamide gels for the purpose. The polyacrylamide gels appear to give better resolution. Vacikova and Blochova (1969) confirmed the finding of a number of variant amylase patterns by agar gel electrophoresis.

In 1969, a horizontal polyacrylamide gel electrophoretic procedure for amylases was described by Boettcher and De La Lande (1969b). Using the technique, they observed patterns similar to those which had been seen by Ogita (1966). Studies on salivary (Boettcher and De La Lande, 1969a) and serum (Boettcher and De La Lande, 1971) amylase isoenzymes revealed several variant types, which appeared to be inherited. They also concluded from their studies that salivary amylases do not occur in serum, and that the isozymes of serum originate in the pancreas. This view was reinforced by observations on a person with very unusual salivary and serum isoamylase characteristics (Boettcher and De La Lande, 1971).

In 1971, Ward *et al.* described a polyacrylamide gel electrophoretic system which, they thought, gave very good resolution of salivary isoamylases. Six to eight bands were observed in specimens from 700 people, and three variant types could be seen. These variant types were shown by family studies to be inherited. At the time, the variants were designated according to the surnames of the people in whom they were observed. In 1973, this group of workers extended their studies of amylase isozymes (Merritt *et al.*, 1973a). Electrophoretic studies were carried out on salivary and pancreatic enzymes in saliva, serum and urine in a number of persons from ethnically distinct populations. Agar gel techniques were compared with the polyacrylamide gel technique. The isozymes of salivary origin were designated "Sa 1" through "Sa 6", in order from origin to anode. Similarly, pancreatic isozymes were denoted "Pa 1" through "Pa 4". A total of 7 salivary phenotypes could be distinguished, each on the basis of an additional isozyme band closer to the origin than (cathodal to) Sa 1, and these were given capital letter designations. In a similar way, three pancreatic amylase phenotypes could be distinguished. The patterns are shown in Figure 37.1. The pedigree data indicated that the amylase isozymes are under the control of two sepa-

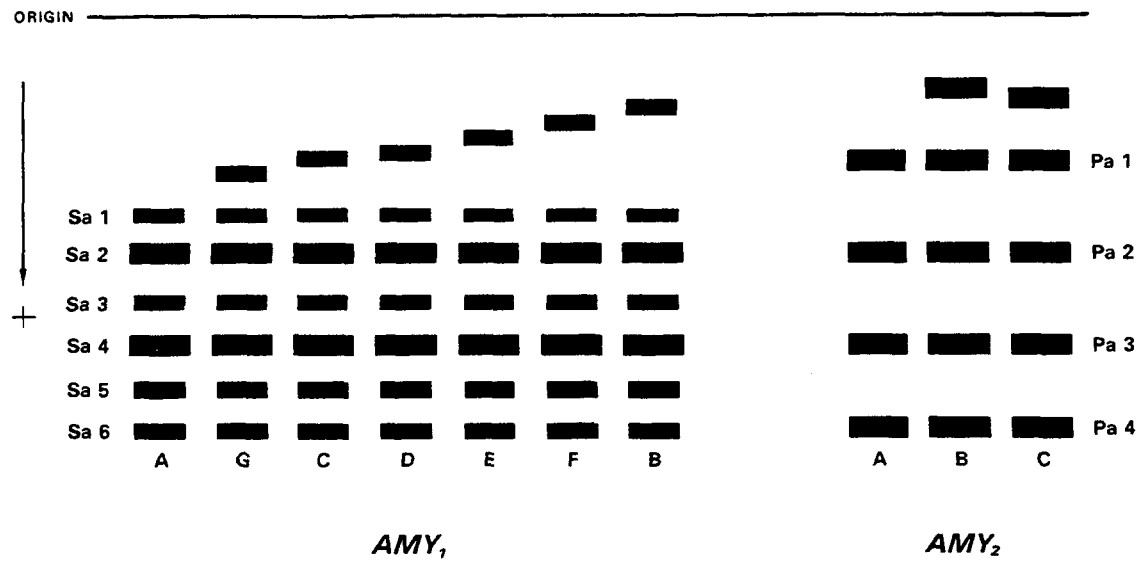


Figure 37.1 Electrophoretic Patterns of *AMY*$_1$ and *AMY*$_2$ Phenotypes following Vertical Polyacrylamide Gel Electrophoresis (after Merritt et al., 1973a)

rate genetic loci, called *AMY*$_1$ and *AMY*$_2$. *AMY*$_1$ codes for the salivary amylases, while *AMY*$_2$ codes for the pancreatic ones. *AMY*$_1$ isozymes are not very strongly expressed, nor very easily detectable in serum or in urine. The readily detectable amylase isozymes in these fluids are products of *AMY*$_2$, and correspond to pancreatic enzyme. In very concentrated specimens, minor components with the mobility of Sa 2 and Sa 4 isozymes of *AMY*$_1$ could be observed. The variant phenotypes could be explained most readily as the expression of heterozygosity of a variant allele with the common allele at the locus. The ordinary (common) alleles are designated *AMY*$_1^A$ and *AMY*$_2^A$. The *AMY*$_1$ variant alleles are designated *AMY*$_1^B$ through *AMY*$_1^G$. *AMY*$_2$ shows a variant type relatively common in Caucasians, and this was called *AMY*$_2^B$, while the other variant, seen only in Blacks, was called *AMY*$_2^C$.

Population studies were conducted on a number of White and Black people in the United States, and on a limited number of Orientals and Nigerians. *AMY*$_1$ variants are uncommon in Caucasians. Only 7 variant phenotypes were seen in 961 people. In Black Americans, however, about 5.3% of 208 people were AMY$_1$ E, and there were several other variants as well. 2 out of 10 Nigerians were AMY$_1$ E. The *AMY*$_1^E$ allele was not observed in Whites. *AMY*$_1^F$ was detected in a Chinese individual. As to *AMY*$_2$ variants, Caucasians show a frequency of about 10% for AMY$_2$ B. Black Americans showed AMY$_2$ B less frequently, but nearly 5% were AMY$_2$ C, a type not seen in Caucasians. These workers recommended that saliva be utilized for AMY$_1$ typing, and that serum or urine be used only for AMY$_2$ typing. It may be noted that *AMY*$_2$ is expressed in seminal plasma (Blake and Sensabaugh, 1976 and 1978), a finding which might find medicolegal applications in time.

The data of Merritt *et al.* (1973a) were consistent with their previous findings (Merritt *et al.*, 1972) that *AMY*$_1$ and *AMY*$_2$ are closely linked. This linkage was not apparent to the Czechoslovakian workers (Kamarýt *et al.*, 1971), but a probable linkage of *AMY*$_1$ with a chromosome 1 marker was noted. Merritt *et al.* (1973b) established that the closely linked *AMY* loci are indeed linked to *Fy* (Duffy blood group locus), thereby assigning *AMY*$_1$ and *AMY*$_2$ to the first chromosome.

37.3.3 Biochemical studies on the amylase isoenzymes

The amylase isozymes of saliva were resolved by polyacrylamide disc gel electrophoresis into 5 to 7 bands by Kauffman *et al.* (1970). Further characterization of the isoenzymes on gel filtration media indicated that they could be arranged into two "families" on the basis of MW. The "A" family consisted of the "odd" bands, 1, 3 and 5, while the "B" family contained 2 and 4, and two forms designated "z". These relationships were found in purified, crystalline preparations of human parotid gland amylase as well (Keller *et al.*, 1971). The "A" family isozymes had MW values of about 62,000, and were glycoproteins (the carbohydrate moiety consisting of 6 moles $GlcNH_2$, 3 moles Fuc, 2 moles Man, and 2 moles Gal per mole glycoenzyme). The "B" family isozymes contained no carbohydrate, and had MW about 56,000. Incubation of the isozymes at pH 9 gave rise to more anionic forms, and the transformation appeared to involve a deamidation reaction. The "A" and "B" family designations of Keller and her colleagues should not be confused with the electrophoretic phenotype designations used by Merritt and his collaborators. The latter group designate the "families" of isozymes as "odds" and "evens". Stiefel

and Keller (1973) purified the pancreatic amylase, and showed that the purified preparation exhibited up to 6 isozymes on polyacrylamide gels. The properties of the enzyme were very similar to but not completely identical with those of the salivary enzymes.

There is good biochemical and immunochemical evidence from the studies of Karn *et al.* (1973, 1974 and 1975) that the complex isozyme patterns exhibited by both AMY_1 and AMY_2 loci can be explained on the basis of post-translational modification of primary gene products. Karn *et al.* (1973) isolated an enzyme from the oral bacterial flora which could convert "odds" to "evens", and it appeared that this "salivary enzyme modifier" protein was responsible for the removal of carbohydrate from the "odd" isozymes, thus yielding the "even" group. The evidence for this and the other studies indicate that some of the primary salivary amylase (AMY_1 locus) product undergoes glycosidation. This modification is followed by deamidation of both glycosylated and nonglycosylated enzyme, and eventually, in whole saliva, glycosylated forms may be deglycosylated. The pancreatic enzyme appears to undergo only deamidation in the course of its post-translational modification. The pancreatic and salivary enzymes are immunologically identical, and all the genetic evidence is consistent with their production by separate, but closely linked, loci, although no crossovers were observed. The most tempting explanation for this situation is that a gene duplication occurred at some point in the evolutionary history of the original *AMY* locus.

37.4 Superoxide Dismutase

Superoxide dismutase [SOD; indophenol oxidase; tetrazolium oxidase; "white patch enzyme"; superoxide:superoxide oxidoreductase; E.C. 1.15.1.1] catalyzes the dismutation of O_2 radicals to yield H_2O_2 and O_2. The enzyme has had a variety of names over the years. Brewer, in 1967, discovered genetic variation in SOD. In the course of staining hundreds of starch gels for various isozymes with PMS-MTT containing mixtures, clear bands in a bluish background had been noted consistently. These "achromatic regions" had always given a similar pattern, until a variable pattern was observed in a single sample. This observation prompted further study, and the protein responsible for the "achromatic regions" turned out to have indophenol oxidase activity. In addition, the "variant" pattern observed turned out to have been inherited.

SOD can be detected in a variety of ways, as Brewer (1967) demonstrated. Assay techniques were elaborated upon by Beauchamp and Fridovich (1971). Most workers put in MTT and PMS and allow the gel to be exposed to light. Sites of SOD activity are clear zones on a blue-purple formazan background. NBT can be used in place of MTT (Beauchamp and Fridovich, 1971). The enzyme also shows a DCPIP oxidase activity in the presence of NADH (Brewer, 1967).

In 1973, Beckman found that certain northern Swedish and some Finnish people showed the variant described by Brewer (1967) in polymorphic frequency. He called the enzyme "superoxide dismutase", and designated the common phenotype as "SOD 1". The variant, seen by Brewer (1967) and relatively frequent (3–4%) in the northern Swedish and Finnish populations, was SOD 2-1. These phenotypes are the result of a pair of alleles, SOD^1 and SOD^2, at an autosomal locus. An example of the rare SOD 2 phenotype has been reported (Beckman *et al.*, 1973a). In 1973, Beckman and his collaborators investigated the tissue distribution and genetic control of human SOD isozymes (Beckman *et al.*, 1973b). All tissues studied had two isozymes, A and B, except red cells and polymorphonuclear leucocytes. The SOD A is a soluble isozyme, and the SOD B originates in the mitochondria. The two forms are controlled by separate genetic loci, SOD_A and SOD_B. No genetic variation has been reported at the SOD_B locus. Red cells lack SOD B, while polymorphonuclear leucocytes lack SOD A. Briggs and Fee (1978) have described the purification and properties of red cell SOD.

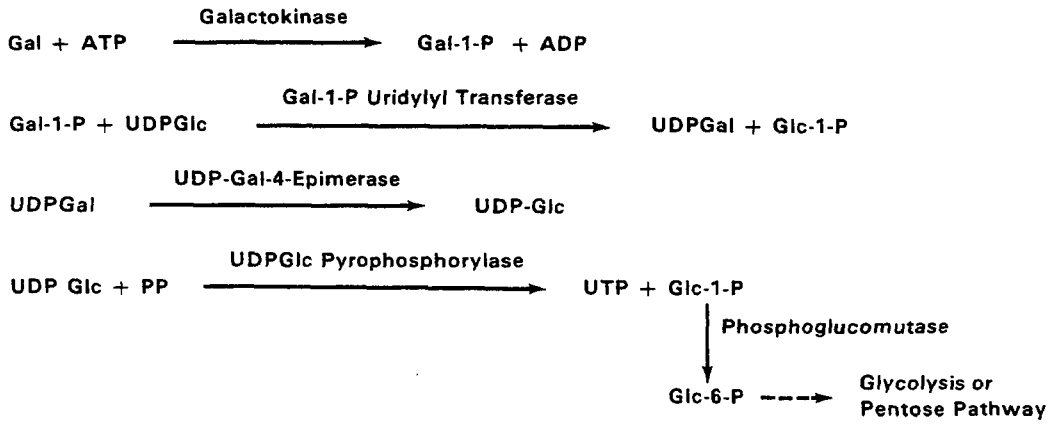
The polymorphism of SOD A does not appear to be very widespread. The only populations with significant frequencies of $SOD_A{}^2$ are the Finnish and northern Swedish (Beckman and Pakarinen, 1973) and the people of Westray (Orkney) (Welch, 1973). No evidence of $SOD_A{}^2$ was seen in Nigerians, nor in Chinese, Japanese, Polynesians and Filipinos in Hawaii (Beckman and Pakarinen, 1973). South African Bantu were all SOD 1 as well (Kirk *et al.*, 1971). There is one report of SOD^2 in three generations of a Japanese family (Shinoda, 1970), and Teng and Lie-Injo (1977) reported a variant in one Filipino in an Asian population survey.

Beckman and Beckman (1975) have reviewed the genetics and function of superoxide dismutase. The SOD_A locus has been assigned to chromosome 21 (Tan *et al.*, 1973) and the SOD_B locus to chromosome 6 (Creagan *et al.*, 1973b; Van Someren *et al.*, 1974).

37.5 Galactose-1-Phosphate Uridylyl Transferase

37.5.1 Metabolic role of the enzyme

Galactose-1-phosphate uridylyl transferase [GALT; uridyl transferase; Gal-1-PUT; hexose-1-phosphate uridylyl transferase; UDP galactose:α-D-galactose-1-phosphate uridylyl transferase; E.C. 2.7.7.12] is one of the key enzymes in the major pathway of galactose metabolism, the net result of which is the conversion of galactose to glucose-1-phosphate. The essential features of this pathway are indicated in Figure 37.2. These reactions were first recognized in microorganisms by Leloir and his collaborators and by Kalckar and his collaborators (Leloir, 1951; Munch Petersen *et al.*, 1953; Kalckar *et al.*, 1953), and the pathway was subsequently found to be operative in mammals as well. Existence of GALT was predicted by Leloir in 1951, and experimentally established by Kalckar *et al.* (1953). Lactose, or milk sugar, is a principal source of galactose in the diet. Lactose is a disaccharide consisting of galactose and glucose residues. Conversion of galactose to



Abbreviations: Gal = Galactose Gal-1-P = Galctose-1-Phosphate
UDPGlc = Uridine Diphosphoglucose UDPGal = Uridine Diphosphogalactose
Glc-1-P = Glucose-1-Phosphate PP = Pyrophosphate Glc-6-P = Glucose-6-Phosphate
ATP = Adenosine Triphosphate ADP = Adenosine Diphosphate UTP = Uridine Triphosphate

Figure 37.2 Principal Reactions of Galactose Metabolism

glucose-1-phosphate allows galactose to be metabolized by way of the glycolytic or pentose phosphate pathways (Figures 37.2 and 33.1).

37.5.2 Genetic variation of GALT

37.5.2.1 Galactosemia. The first kind of genetic variation described for GALT was a deficiency syndrome, known as galactosemia. The condition is a classical "inborn error of metabolism" (see section 1.2.2.1). The existence of galactosemia has been recognized for many years. In 1935, Mason and Turner described extensive studies on a patient affected with it, and they recognized that the normal metabolic pathway of galactose metabolism was affected in some way, and that withholding galactose from the diet was effective in reducing the severity of the clinical consequences. It was not until 1956, however, that Isselbacher *et al.* demonstrated conclusively that the condition represented a deficiency of GALT. Galactosemia is inherited as an autosomal recessive characteristic, and it is characterized by cirrhosis, cataract, failure to grow and develop normally, and mental retardation. The clinical consequences are the result of toxicity of galactose or of one of its metabolites. Although the exact nature of the pathogenesis is not fully clear, accumulation of galactitol and galactose-1-phosphate have been implicated as being responsible. The clinical and genetic aspects of galactosemia have been well reviewed by Segal (1978). The gene for galactosemia is now designated $GALT^{G}$ and the common allele as $GALT^{A}$. In the past, it has been designated *gt* or Gt^{G}, while the common allele was designated Gt^{+}.

37.5.2.2 Further genetic variation of GALT. In 1965, Beutler *et al.* found another genetic variant of GALT. This variant was detected as the result of studies on screening tests for galactosemia heterozygotes. In classical galactosemia, since it is recessive, homozygotes are afflicted with the condition. The heterozygotes are asymptomatic, but their red cell GALT activity is on the order of one-half that normally seen. Beutler *et al.* (1965a) encountered some people in their family studies who had enzyme activity levels significantly higher than expected for heterozygous carriers of galactosemia, and yet significantly lower than that seen in the general population. These activity levels were inherited, and the phenotype was described as the "Duarte variant". The gene responsible was designated Gt^{D}, and it is now usually called $GALT^{D}$. It has sometimes been called Gt^{2}, where Gt^{1} then denotes the normal allele (Kühnl *et al.*, 1974b). It remained to be established that the Duarte variant gene was an allele of the galactosemia gene. An informative family, in which both genes were segregating, served to establish the allelic relationship between $GALT^{C}$ and $GALT^{D}$ (Beutler *et al.*, 1966a). The $GALT^{D}$ allele gives rise to a lower than usual activity form of Gal-1-P uridylyl transferase, and Duarte heterozygotes ($GALT^{A}GALT^{D}$) exhibit about 75% of the usual enzyme activity. Duarte homozygotes ($GALT^{D}GALT^{D}$) exhibit about 50% activity levels. Heterozygotes for galactosemia ($GALT^{A}GALT^{G}$) also exhibit about 50% normal activity levels, whereas galatosemics ($GALT^{G}GALT^{G}$) have no activity. People heterozygous for both galactosemia and Duarte genes ($GALT^{D}GALT^{G}$) have about 25% normal levels of enzyme activity. Duarte variants are healthy, and the enzyme they make is similar to many of its properties to the normal one (Beutler *et al.*, 1966b).

In 1965, Beutler and his collaborators found that the

Duarte variant GALT was electrophoretically distinguishable from the normal enzyme on starch gels, exhibiting a slightly greater anodic mobility (Beutler *et al.*, 1965c; Mathai and Beutler, 1966). This finding enabled a number of workers to confirm the genetic hypothesis by means of population and family studies (Bissbort and Kömpf, 1973b; Kühnl *et al.*, 1974; Martin and Kienzler, 1975). Improvements in the electrophoretic and staining procedures were also forthcoming (Bissbort and Kömpf, 1973a; Ng *et al.*, 1969).

In 1973, Ng *et al.* reported another variant of GALT, which was called the "Los Angeles" variant. This phenotype was characterized by normal to higher than normal enzyme activity, and an electrophoretic pattern very similar to that of the Duarte variant. The differences in intensities of the triplet band patterns were sufficient to distinguish the two variants (see in Figure 37.3), but Ng *et al.* (1973) said that "Los Angeles" homozygotes were difficult to distinguish from "Los Angeles/Duarte" heterozygotes by electrophoresis. Sparkes *et al.* (1977) reported an improved electrophoretic procedure which, they said, made it easier to distinguish 'Los Angeles' and 'Duarte' variants.

A rare variant of GALT, designated the 'Berne' variant, was described in a Swiss population by Scherz *et al.* (1976). Only three people with this phenotype were seen in a survey of 1,668 persons.

Two other variants of GALT have been reported, both exceedingly rare. In 1969, Schapira and Kaplan described two sibs in a French family who suffered from clinical galactosemia, but who exhibited an incomplete enzyme deficiency. Electrophoresis revealed an enzyme with a slower anodal mobility than normal GALT, and thus, apparently, a structurally different form. This variant was called "Rennes". The structurally different enzyme could also be demonstrated in cultured fibroblasts (Hammersen *et al.*, 1975). In 1971, Chacko *et al.* found an unstable form of GALT in a patient with clinical galactosemia. In fresh material, the enzyme was about 40% as active as normal enzyme, but it lost activity rapidly upon storage. It had an electrophoretic mobility slower than normal GALT, but was different from 'Rennes', and was called the "Indiana" variant. Family studies indicated that the proband in this study was heterozygous for the 'Indiana' variant allele and the classical galactosemia allele ($GALT^{G}$).

37.5.3 Population studies on the GALT variants—A further type of galactosemia and a GALK variant

There is, and has been, considerable clinical interest in galactosemia syndromes. It is important to detect the condition in newborns as soon as possible, so that treatment can be initiated; further, from a genetic counseling point of view, it would be desirable to be able to detect heterozygotes. Screening and population studies on GALT have often been carried out with these objectives in mind.

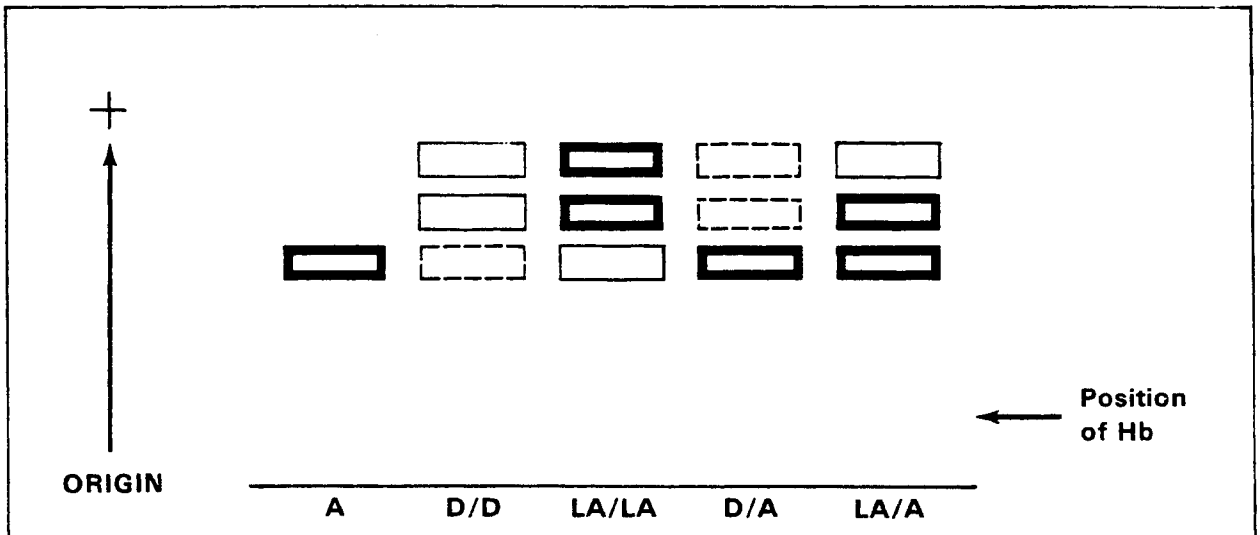


Figure 37.3 Electrophoretic Patterns of GALT Variants (after Ng et al., 1973)

Beutler (1969b) has reviewed the assay techniques for GALT used for screening hemolysates for clinically significant GALT deficiency syndromes. Until the recognition of the Duarte variant, screening tests based on activity determinations gave misleading results. Duarte homozygotes show about 50% normal activity levels, while heterozygotes show about 75%. Likewise, heterozygotes for galactosemia show about 50% activity. Duarte homozygotes are indistinguishable from galactosemia heterozygotes, therefore, on the basis of enzyme assay tests alone. The Duarte allele occurs with a much higher frequency in many populations than the galactosemia gene, and estimates of the frequency of $GALT^G$ must be corrected for the $GALT^D$ in the gene pool. The Duarte allele is relatively common, some 8% to 15% of the population being heterozygous for it (Beutler *et al.*, 1965b; Mellman *et al.*, 1968; Martin and Kienzler, 1975). The 'Los Angeles' variant is less frequent, occurring in about 4% to 5% of the population (Ng *et al.*, 1973). The galactosemia gene ($GALT^G$) occurs with a frequency of about 0.002 to 0.005. It is thus expected that about 0.4% to 1.0% of the population would be heterozygous for galactosemia, and that galactosemia should occur in about 1/40,000 to 1/250,000 people. In actual fact, the incidence of galactosemia has been recorded as 1/70,000 in England, 1/35,000 in New York State and 1/190,000 in Massachusetts (Segal, 1978). This material has been reviewed by Beutler and Mathai (1968).

Gitzelmann (1965 and 1967) described several patients with galactokinase (GALK) deficiency, which had been inherited (see in Figure 37.2). These patients were older, and the principal clinical manifestation of the syndrome seemed to be the development of cataracts, even in the earliest years. The association of this syndrome with cataracts seems established, but relationships to other abnormalities are not very clear. In 1975, Tedesco *et al.* conducted a survey of 1,700 pregnant women in the Philadelphia area for GALT and GALK activities. A variant of GALK, which leads to lower activity of the enzyme, was found and named the 'Philadelphia' variant. It was relatively common in Black, but rare in White people. The responsible allele was designated $GALK^P$. Segal (1978) suggested that classical galactosemia be called "transferase deficiency galactosemia", and that the syndrome described by Gitzelmann (1965 and 1967) be called "galactokinase deficiency galactosemia".

37.5.4 Other notes on GALT

Tedesco (1972) purified the GALT from liver and red cells, and compared the properties of normal, Duarte and galactosemia gene products. They were all similar, and had MW about 90,000. Sodium dodecyl sulfate polyacrylamide gel electrophoresis suggested that the enzyme might be a trimer. All three showed immunological identity with a liver anti-GALT antibody. The antibody could also be used to demonstrate that galactosemic individuals make a protein which is immunologically indistinguishable from the normal one, but catalytically inactive. Marcus *et al.* (1977) carried out biochemical studies on the reaction mechanism with purified human red cell enzyme. Williams (1978) purified red cell GALT to a higher degree. The enzyme had a MW of 67,000 as estimated by Sephadex chromatography, but of 88,000 by ultracentrifuge studies. It consists of two similar subunits with MW 44,000.

No reports on the typing of GALT in bloodstains were found in the literature. Hammersen and Levy (1977) showed, however, that blood dried on filter paper could be used for activity and electrophoretic screening measurements. The enzyme is thus stable, at least in freshly dried blood. The Duarte variant occurs with a high enough frequency to make GALT a useful genetic marker, if suitable procedures were available for typing the enzyme in bloodstains.

Eriksen and Dissing (1980) have recently described the application of the GALT system to disputed parentage cases. They denoted the GALT genes $GALT^1$ (usual; normal) and $GALT^2$ (representing both $GALT^D$ and $GALT^{Los\ Angeles}$). Treating the system in this way resulted in a probability of exclusion for a true non-father of about 6.6%, based on gene frequencies determined in over 2,000 Danish people. On apparent mother-child incompatibility in the studies was most easily explained by the presence of an inactive allele at *GALT*.

37.6 Glucose Phosphate Isomerase

Glucose phosphate isomerase (GPI; glucose phosphate isomerase; phosphohexose isomerase; D-glucose-6-phosphate ketol-isomerase; E.C. 5.3.1.9) catalyzes the reversible interconversion of D-glucose-6-phosphate and D-fructose-6-phosphate. The enzyme has been shown to exhibit genetic variation, but the variants are all extremely rare. The enzyme survives well in dried blood, and can be typed in bloodstains (Culliford, 1971). It is a very poor genetic marker, however, because variants are observed so seldom.

In 1968, genetic variation in GPI was reported almost simultaneously by Fitch *et al.* and by Detter *et al.* Fitch *et al.* (1968) found five different phenotypes in a survey of 1,650 people. Detter *et al.* (1968) surveyed 3,397 people, and identified 11 phenotypes, which could be accounted for by 10 alleles at an autosomal locus. These are now usually called GPI^1 through GPI^{10}, and GPI 1-1 is by far the most common type. Only 20 people of the 3,397 surveyed were other than GPI 1-1. Welch (1971) said that eight variants had been seen in 3,300 bloods, and this total probably included the data of Fitch *et al.* (1968). Blake and Omoto (1972) identified nine GPI variants in a survey of 4,000 people from various populations in Asia and Oceania.

Quantitative variation has also been observed with GPI. In 1968, Baughan *et al.* found a patient suffering from congenital nonspherocytic hemolytic anemia, and he was found to have a GPI deficiency. The enzyme deficiency was inherited as an autosomal recessive, and the heterozygotes in the family showed intermediate levels of enzyme activity. A number of cases of GPI deficiency associated with hemolytic anemia have since been reported. Some of these have

been given trivial names based on the cities of origin of the possessors, while others have not. Some variant types have altered electrophoretic mobility, while others do not. Sixteen variants were listed in the review of GPI deficiency by Paglia and Valentine (1974). Welch (1971) reported two half activity variants, called 'Cowen' and 'Hay', the former of which showed altered electrophoretic mobility. Rotteveel *et al.* (1974) reported a variant child in a Dutch family who had hemolytic anemia, and they called the phenotype 'Nijmegen'. Family studies indicated that this person was heterozygous for two different rare PGI alleles. Family members who were heterozygous for one or the other of these exhibited intermediate levels of enzyme activity but not hemolytic anemia.

It may be noted that GPI is present in seminal plasma (Blake and Sensabaugh, 1976 and 1978), and in significant quantitites, approaching its concentration in blood on a per volume basis. The enzyme would be expected to be readily detectable in semen, therefore, but its value as a genetic marker is very limited because none of the variant alleles occur in significant frequencies in major populations studied thus far.

37.7 Glutathione Reductase

Glutathione reductase (GSR; NAD(P)H:oxidized-glutathione oxidoreductase; E.C. 1.6.4.2) catalyzes the reduction of oxidized glutathione by NADPH to yield reduced glutathione and NADP (see Figure 33.1). In 1967, Long reported a GSR variant characterized by a greater anodal mobility than the common type. The variant was found to be relatively common in Blacks, 6 homozygotes being found among 196 subjects. The variant is inherited in a straightforward autosomal, codominant way. Kaplan (1968) confirmed these findings. The gene frequency for the variant allele in American Blacks is about 0.13. The electrophoretic system consisted of 0.1M Tris-HCl, pH 9.6, containing 4.5 mM EDTA for bridge buffer, and a 1:10 dilution of this solution for the gel. The GSR is detected by adding an overlay containing GSSG, NADPH, DCPIP and MTT in 0.25M Tris-HCl buffer, pH 8.4. The reaction mixture also detects NADPH diaphorase (section 37.9). The diaphorase runs well ahead of the GSR, however, and can be located by omitting the GSSG from the staining reaction mixture.

There are a number of reports in the literature on inherited GSR deficiency, associated with hemolytic anemia, and sometimes with other disorders. This subject was reviewed by Waller (1968) and by Brewer (1969). The deficiency syndromes are inherited autosomally (Blume *et al.*, 1968), and heterozygotes have intermediate levels of GSR activity in red cells, white cells and platelets.

GSR has been purified quite extensively. It is a flavoprotein with a MW of 100,000, and the active enzyme is a dimer. A low resolution x-ray structure has been determined (Zappe *et al.*, 1977).

37.8 Glutathione Peroxidase

Glutathione peroxidase (GPX; glutathione:hydrogen-peroxide oxidoreductase; E.C. 1.11.1.9) catalyzes the oxidation of reduced glutathione by H_2O_2 and certain organic peroxides (see Figure 33.1). It is perhaps the only known enzyme having selenium as an essential cofactor (Godwin, 1975).

Studies by Beutler and West (1974) and Beutler *et al.* (1974) disclosed an electrophoretic variant of GPX having faster (more anodal) mobility than the usual form. Electrophoresis was carried out on starch gels in 0.1M citric acid-phosphate, pH 7.5, buffer diluted 1:10 for the gel. The enzyme zones were detected in an overlay of t-butyl hydroperoxide, GSH, NADPH, EDTA and glutathione reductase in phosphate buffer, pH 7, and were represented as defluorescent bands on an otherwise fluorescent background under UV light. Hydrogen peroxide cannot be used in the detection reaction on gels because the spontaneous (nonenzymatic) oxidation reaction rate is too greater. The electrophoretic variant, called the 'Thomas' variant, is relatively common in Blacks, but rare in Whites. About 6.4% of 392 Black blood donors were 'Thomas' heterozygotes, while less than 1% of 388 Caucasian donors showed the variant phenotype. No variants were seen in a small number of Orientals. "Storage" bands can develop in hemolysates, but the ones that could interfere with the diagnosis of the Thomas phenotype are removed by mercaptoethanol treatment. The Thomas variant is inherited as an autosomal codominant allele of the usual GPX gene. Kelly and Schedlbauer (1978) reported that GPX activity could be assayed in dried blood, indicating that the enzyme is stable at least in freshly dried stains.

In 1975, Beutler and Matsumoto reported that GPX levels of activity in red cells varied in systematic ways in certain populations. The lower activity enzyme, characteristic of Jewish and Mediterranean populations, had different thermostability characteristics, and may represent the expression of another allele of GPX, characterized by decreased RBC activity.

37.9 Hexokinase

Hexokinase (HK; ATP:D-Hexose-6-phosphotransferase; E.C. 2.7.1.1) may be regarded as the first enzyme in the Embden-Meyerhof pathway (glycolysis), and catalyzes the phosphorylation of glucose at the expense of ATP to yield ADP and Glc-6-P (Figure 33.1). Mannose and glucosamine can also act as substrates for some of the hexokinases.

Red cell HK exhibits a series of isoenzymes, their number and characteristics being dependent on the methods employed for electrohoresis and staining (Eaton *et al.*, 1966; Holmes *et al.*, 1967; Brewer and Knudsen, 1968; Holmes *et al.*, 1968; Schröter and Tillman, 1968).

Four distinct kinds of HK have been found in rat tissues, and these have been distinguished on the basis of various properties. Different tissues vary in the distributions of the different kinds (Katzen and Schimke, 1965). Four kinds of HK may be distinguished in human tissues as well, and they are called HK I through HK IV. HK IV has the greatest anodal electrophoretic mobility. HK IV has a high K_m for glucose, while the other three have relatively low K_m for it (Rogers *et al.*, 1975; Povey *et al.*, 1975). A variant kind of

HK III was found in fresh leucocytes (Povey *et al.*, 1975) and the data indicated that HK III was determined by an independent locus HK_3. The variant allele was designated HK_3^2, and 10 of 330 English people were HK_3 2-1. No genetic variants of HK I or HK II were found in a survey of 800 Europeans (Rogers *et al.*, 1975), but it is thought likely that they are under the control of separate genetic loci.

Patients with congenital hemolytic anemia who lack red cell HK have been reported (Valentine *et al.*, 1967 and 1968). The deficiency appeared to be inherited as an autosomal recessive characteristic.

37.10 Lactate Dehydrogenase

Lactate dehydrogenase (LDH; lactic dehydrogenase; L-lactate:NAD^+ oxidoreductase; E.C. 1.1.1.27) was one of the first enzymes found to exhibit multiple molecular forms, and the early studies on LDH isoenzymes represented the beginning of an explosive stage of growth and development in biochemical genetics. This aspect of LDH was discussed in section 26. Genetic variation of LDH is fairly rare in most populations, and it is not a very useful population marker. The variation in tissue distribution of LDH has led to its application as a specific tissue marker in medicolegal identification problems. These applications were discussed in sections 8.1.5 and 10.8.

Native LDH isozymes are tetramers, consisting of two pairs of nonidentical subunits. The separate subunits are under the control of different autosomal genetic loci, called LDH_A, LDH_B and LDH_C. LDH_C codes for LDH-X of spermatozoa (section 10.8) and the structure of LDH-X may be designated C_4. LDH isozymes in other tissues are the result of random associations of A and B subunits: B_4, AB_3, A_2B_2, A_3B and A_4. These isoenzymes are designated LDH-1 through LDH-5, respectively. The relative amounts of LDH_A and LDH_B products vary from tissue to tissue, so that various LDH isozymes tend to predominate in certain tissues. In liver and skeletal muscle, for example, LDH-5 predominates, whereas LDH-1 and LDH-2 are the major isozymes of cardiac muscle.

Allelic variants at both loci are known. They are detected as multiplicity of bands after electrophoretic separation. The large number of bands can be explained as follows: An individual heterozygous for a variant allele at LDH_B makes B subunits as well as "variant B" subunits (let us call these "β"). Such a person can make 15 separate isozymes of LDH, i.e. B_4, $B_3\beta$, $B_2\beta_2$, $B\beta_3$, β_4, B_3A, $B_2\beta A$, $B\beta_2 A$, $\beta_3 A$, B_2A_2, $B\beta A_2$, β_2A_2, BA_3, βA_3 and A_4. A heterozygote for an LDH_A locus variant can likewise make 15 different isoenzymes. The isoenzymes seen with a particular variant depends on the electrophoretic system and on the tissue examined.

In 1963, Nance *et al.* reported the first A variant in a Brazilian family. Boyer *et al.* (1963) found the first B variant in a young Nigerian man. Kraus and Neely (1964) described four variants, one of which was similar to that of Boyer *et al.* (1963). These were found in people from Memphis, and were designated LDH-A_{Mem-1}, LDH-A_{Mem-2}, LDH-B_{Mem-3} and LDH-B_{Mem-4}. Vessell (1965) described a homozygous B variant. An A variant was described in an English population by Davidson *et al.* (1965). Blake *et al.* (1969) examined a New Guinea population and found a further A variant called 'New Guinea'. Several additional variants have been described in Asian Indian populations where, in certain caste groups, they can reach frequencies of 3% to 4% (Ananthakrishnan *et al.*, 1970; Das *et al.*, 1972). A B-variant similar to 'Madras-1' and 'Memphis-3' has been described in a person from Sofia, Bulgaria (Ananthakrishnan *et al.*, 1972). Lie-Injo *et al.* (1973) described some additional variants in Kuala Lumpur, Malaysia.

Kitamura (1971) found a 64 year old Japanese man with a complete deficiency of LDH-B. The condition was inherited, and this interesting patient made only LDH-5 (A_4) isoenzyme.

37.11 Pepsinogen (Pg)

Pepsinogens are precursors of the gastric digestive enzyme pepsin. This particular protein is not uniquely clasified by the Enzyme Commission, but would be in the 3.4.23 sub-sub-class. This interesting polymorphism in Pg is mentioned briefly because some of the isozymes are excreted in urine, and urine may be used to determine the phenotypes. The urine has to be concentrated about 20-fold, in order to detect the Pg isozymes, and it may or may not be possible to devise procedures applicable to the kinds of samples encountered in medicolegal cases. Nevertheless, urine is sometimes encountered, and this polymorphism is well distributed in the population.

Samloff (1969) showed that 8 bands of protease activity with an acid pH optimum can be resolved from different sections of gastric mucosal tissue. These bands are called Pg 1 through Pg 7, with Pg 1 having the most anodal mobility. The eighth, slowest zone is probably cathepsin. Pg 1 through 5 constitute one group of isozymes, while Pg 6 and Pg 7 make up a second group. Pg 2 through Pg 5 of the first group are consistently seen in urine, while Pg 1 is seen variably (Samloff and Townes, 1970a). The Pg 2–Pg 5 isozymes exhibit a polymorphism consisting of two phenotypes, A and B. In A, the Pg 5 is present, whereas in B, it is absent (Samloff and Townes, 1970b; Samloff *et al.*, 1973). Type B was estimated to occur in about 14% of U.S. Whites and Blacks, suggesting a gene frequency for Pg^A of 0.62 and about 0.38 for Pg^B. Townes and White (1974) said they had detected another phenotype in Blacks, called B^1. They also said that, in a sample of nearly 500 U.S. Blacks, about 22% were Pg B, while about 4% were Pg B^1. Bowen *et al.* (1972) presented evidence for another allele, Pg^C. Pg^C homozygotes had a strong Pg 4 band with a small amount of activity in Pg 3. In a sample of 424 Canadian schoolgirls, Pg^C occurred with a frequency of about 0.03. The genetics of the Pg system may actually be somewhat more complicated than the initial studies have suggested thus far (Weitkamp and Townes, 1975). Samloff and Liebman (1972) showed that Pg can be detected in semen, and that the seminal Pg is identical with Pg 6 and Pg 7. Antisera prepared against this

material, or against the Pg 6 and Pg 7 fractions of gastric mucosa do not cross react with the Pg 2 through Pg 5 fractions. It is quite likely that the two groups are under separate genetic control.

37.12 Uridine Monophosphate Kinase

Uridine monophosphate kinase (UMPK) catalyzes the phosphorylation of UMP by ATP to yield UDP and ADP. This enzyme belongs to the Enzyme Commission sub-subclass 2.7.4. UMPK is one of the newest polymorphic enzymes to be described in human red cells.

Giblett *et al.* (1974) found four electrophoretic phenotypes of the enzyme, which were called UMPK 1, 2-1, 2 and 3-1. Family studies indicated that these represented the expression of three codominant alleles at an autosomal locus, $UMPK^1$, $UMPK^2$ and $UMPK^3$. $UMPK^2$ occurs with a frequency of about 0.045 in U.S. Caucasians, 0.011 in U.S. Blacks and 0.071 in U.S. Orientals. The $UMPK^3$ allele is rare, except in the Cree Indians. The $UMPK^2$ isozyme has only about 1/3 the activity of the $UMPK^1$ product. There is a possibility that homozygosity for $UMPK^2$ may be associated with some defect in the immune response. The *UMPK* locus may have a linkage relationship with *Rh* or with *Sc* (Scianna blood group locus), but further study will be needed to be certain about it (Giblett *et al.*, 1975). Toyomasu *et al.* (1977) have described a fourth allele of *UMPK*, $UMPK^4$, which was detected as a 4-1 in a survey of 770 Japanese in Osaka. Teng *et al.* (1976) have purified the UMPK 1 and UMPK 2 enzymes and examined their properties. They were similar except for the greater thermolability of UMPK 2. Gallango *et al.* (1978) described the partial purification and characterization of the enzyme from an UMPK 3 homozygote. It differed in pH optimum and thermal stability, but had similar kinetic characteristics with the UMPK 1 and UMPK 2.

37.13 Diaphorases

"Diaphorase" is a trivial name for enzymes which catalyze the reduction of an acceptor by NADH or NADPH, or by both of them. These enzymes are classified in the 1.6 subclass by the Enzyme Commission. The sub-sub-class is dependent upon the nature of the acceptor. If the physiological acceptor is not known, the NADH-dependent enzymes are classified 1.6.99.3, while the NADPH-dependent enzymes are classified 1.6.99.1.

It is clear from the work of Fisher *et al.* (1977a) that the human diaphorases are determined by at least three separate genetic loci, which have been called DIA_1, DIA_2 and DIA_3. The products of the first two of these loci occur in red cells and in a number of tissues. The DIA_1 enzymes are NADH-dependent, while those of DIA_2 are NADPH-dependent. The products of DIA_3 occur in a number of tissues, but not in red cells. The DIA_3 locus is significantly polymorphic in human beings, and this fact was discovered by Caldwell *et al.* (1976) in spermatozoa. The isozymes determined by DIA_3 were, thus, first called 'sperm diaphorases' (SD).

37.13.1 DIA_1 and DIA_2

The enzyme determined by the locus now called DIA_1 was classically called NADH-methemoglobin reductase. Since methemoglobin does not bind oxygen, it is important to the well being of mature red cells and to the economy of the organism possessing them that methemoglobin concentrations not be allowed to reach very high levels. Pathologically excessive levels of methemoglobin in circulation characterize a condition known as methemoglobinemia. Many cases of congenital methemoglobinemia have been described, and it is now clear that the condition represents, in many cases, an inherited deficiency of NADH-dependent methemoglobin reductase activity. Mental retardation often accompanies the clinical picture seen in the condition as well. A number of variant types of NADH diaphorases are associated with the deficiency syndromes, some of which show altered electrophoretic mobility (Kaplan and Beutler, 1967; West *et al.*, 1967; Bloom and Zarkowsky, 1969; Hsieh and Jaffe, 1971). These have been named after the cities in which they were first observed. Leroux and Kaplan (1972) discovered that the NADH diaphorase occurs in many tissues besides red cells, including leucocytes, platelets, brain, muscle, liver and placenta. This finding suggested that the enzyme has some broader physiological significance than its methemoglobin reductase activity, since there is no hemoglobin in these other tissues. Hultquist and Passon (1971) presented experimental evidence that the NADH diaphorase is probably an NADH-cytochrome b_5 reductase, and that the reduction of methemoglobin in the red cell by NADH is mediated by cytochrome b_5. In this view, the enzyme catalyzes the reduction of oxidized cytochrome b_5 by NADH, and the reduction of methemoglobin by reduced cytochrome b_5 is spontaneous. These results help to provide a tidy explanation for the presence of the enzyme in nonerythroid tissues, and account for the origin of the current systematic name of the enzyme: 'cytochrome b_5 reductase'; NADH:ferricytochrome b_5 oxidoreductase; E.C. 1.6.2.2. Leroux *et al.* (1975) have studied the red cells and tissues of a number of victims of congenital methemoglobinemia, and have found that the enzyme defect is a generalized one, not restricted to the red cells. The fact that the enzyme occurs in brain tissue may help to explain why mental retardation is seen in connection with the deficiency syndromes (Leroux *et al.*, 1975). Leroux *et al.* (1977) have carried out further studies on the enzyme which suggests that, at least in terms of immunological identity, the red cell 'NADH methemoglobin reductase' and the 'cytochrome b_5 reductase' activities in red cells and tissues are the result of the same protein. A microsomal form of the enzyme from placental tissue, which could be solubilized by treatment with deoxycholate, was similar but not quite identical to the soluble forms of the enzyme. The subject of NADH diaphorase variation in the deficiency syndromes has been well reviewed by Scott (1968), Huennekens *et al.* (1968) and Jaffé (1969). Ross (1963) showed that cord blood contains significantly lower diaphorase activity than adult control blood, and said that

this was probably a major cause of methemoglobinemia in newborn infants. In the majority of cases, however, the condition is transient.

Red cell NADH diaphorse also exhibits rare inherited variants, which are not associated with methemoglobinemia. Brewer *et al.* (1967b) described a probable variant. In 1970, Detter *et al.* found two examples of a variant DIA among 378 donors. This variant enzyme was definitely inherited in an autosomal codominant fashion (Detter *et al.*, 1970a). In 1970, Hopkinson *et al.* screened over 2,700 people for electrophoretic DIA variants, and could identify six rare phenotypes in addition to the usual one, and these could be accounted for by the heterozygous expression of five rare DIA alleles, designated DIA^2 through DIA^6, where DIA^1 is the usual allele. About 1% of Europeans, Blacks and Asiatic Indians showed variant DIA phenotypes. Tariverdian *et al.* (1970a) found two variant phenotypes in four people out of 725 in Germany. They called their variant types '2–1' and '3–1'. Williams and Hopkinson (1975) screened a further 3,060 Australians, and found a new phenotype, called DIA 7–1, due to a seventh allele, DIA^7. They said that the '2–1' and '3–1' of Tariverdian *et al.* (1970a) probably corresponded to the 2–1 and 4–1 types of Hopkinson *et al.* (1970). Williams and Hopkinson (1975) knew of 12,608 people who had been phenotyped for red cell DIA, and only 103 were other than DIA 1. This number corresponds to a frequency of about 8.2/1,000 for all DIA variant phenotypes combined.

There was evidence from the work of Scott *et al.* (1965) that the red cell possessed an NADPH-dependent diaphorase, and that this was a separate enzyme activity from the NADH-dependent one. Kaplan and Beutler (1967) presented additional experimental evidence that the NADPH-dependent enzyme was indeed distinct. There are no reports of genetic variation in NADPH diaphorase, except for one family in which a deficiency of the enzyme was noted (Sass *et al.*, 1967). The deficiency was detected in a Black man, who had no hematological abnormalities and only a slight anemia. The condition was found in five other members of the family, however, and was clearly inherited. The fact that these deficient people were healthy lends strength to the belief that the majority of methemoglobin reductase activity in the red cell is attributable to 'NADH methemoglobin reductase', i.e., to the cytochrome b_5 reductase discussed in the foregoing paragraphs.

In 1977, Fisher *et al.* conducted an extensive study of the diaphorase activities of red cells and a number of different tissues. Their results (Fisher *et al.*, 1977a), coupled with the data from other studies that have already been discussed, indicated that the NADH-dependent and the NADPH-dependent diaphorases were under the control of separate genetic loci. The loci were designated DIA_1 and DIA_2. Thus, DIA_1 is responsible for the cytochrome b_5 reductase activity, which was called NADH methemoglobin reductase for a number of years. DIA_2 codes for the NADPH-dependent enzyme. They showed further, that a third kind of diaphorase found in human tissues, and discussed in section 37.13.2, is controlled by yet another genetic locus, which was designated DIA_3. The data of Fisher *et al.* (1977a) indicated that the DIA_1 isozymes had a MW of about 31,000, while those of DIA_2 were about 18,000 daltons. The DIA_2 enzyme was more thermostable than the DIA_1 product, and the two differed by 2 pH units in isoelectric point, DIA_2 enzyme being the more acidic.

Studies with human-rodent hybrid cell lines have resulted in the assignment of DIA_1 to chromosome 22 (Fisher *et al.*, 1977b).

37.13.2 DIA_3 (Sperm Diaphorase)

In 1976, Caldwell *et al.* discovered that an enzyme which had diaphorase activity, and which occurred in human spermatozoa but not in seminal plasma, exhibited three different electrophoretic patterns in 52 primarily Caucasian men. Population analysis strongly suggested that this polymorphism had an autosomal codominant genetic basis, and the pair of alleles throught to be responsible were designated SD^1 and SD^2 (for *S*perm *D*iaphorase). Family studies as such could not be done at the time. The phenotypes, which were detected on polyacrylamide gels, were called 1, 2–1 and 2, and were expressed in testicular tissue as well. The polymorphism was well distributed in the limited sample of people typed, SD^1 having a frequency of about 0.71.

Kühnl *et al.* (1977b) confirmed these findings in the Hessen population. SD was typed on thin agarose gels. They reported two additional phenotypes, which could be attributed to a third allele, SD^3, and the gene frequencies in 141 men were found to be $SD^1 = 0.76$, $SD^2 = 0.22$ and $SD^3 = 0.02$. Kühnl *et al.* (1977b) found that SD could be phenotyped in ovarian and oviduct tissues, where the polymorphism is also expressed. The enzyme is thus not restricted to male tissue, and the German workers said that 'sperm diaphorase' was probably not a good name for the enzyme.

Fisher *et al.* (1977a), in their extensive survey of diaphorases in many tissues, demonstrated that the 'sperm diaphorase' was not restricted even to gonadal tissues, but that it occurred in a number of human tissues. There was every indication from the data that this diaphorase was under the control of a distinct genetic locus, and they said that the most logical designation for the locus would be DIA_3. The alleles formerly designated SD^1, SD^2 and SD^3 thus become DIA_3^1, DIA_3^2 and DIA_3^3. The DIA_3 isozymes can oxidize both NADH and NADPH, and are not identical to a cytochrome b_5 reductase. DIA_3 enzymes were found to have a MW of about 31,000, similar in this respect to DIA_1 enzyme. The enzyme from DIA_3^1 homozygotes had an isoelectric point of 7.9. In 1979, Edwards *et al.* carried out studies on the biochemical properties of the DIA_3 isozymes. An improved starch gel electrophoresis system was described in this work. The isozymes determined by DIA_3^1 and DIA_3^2 were found to be quite similar in terms of thermostability, affinity for Blue Sepharose and slow anodal electrophoretic mobility in borate containing buffers, while the DIA_3^3 product differed markedly from the other two in

these respects. The gene frequencies for the three alleles in 346 English people were almost identical to those seen by Kühnl *et al.* (1977b) in Germany.

The phenotypic distribution of *DIA*$_3$ types make the system a potentially valuable genetic marker. It may be a particularly valuable marker in cases of sexual assault in which sufficient sperm cells could be recovered. This possibility was suggested by Caldwell *et al.* (1976) and by Gladkikh (1978). The latter investigator detected *DIA*$_3$ phenotypes in seminal stains, and the gene frequencies reported in his sample of 86 people in the U.S.S.R. were very similar to those observed by Caldwell *et al.* (1976), and by the European workers. Suyama *et al.* (1979) reported frequencies of $DIA_3^1 = 0.84$ and $DIA_3^2 = 0.16$ in a sample of 54 Japanese. Oepen *et al.* (1980) noted that *DIA*$_3$ isozymes could be determined in seminal stains up to 4 weeks old, and in some stains up to 6 weeks old. They discussed various technical problems with the typing systems as well. There were too few DIA$_3$ 1 types in their material (n = 32) as compared with the frequencies seen by other authors, and they said that 6 specimens could not be typed for the isoenzymes. Other investigators have reported DIA_3^1 frequencies ranging from 0.71 to 0.84 in various populations. Oepen *et al.* (1980) quoted Kopetz *et al.* (1979) as having typed 325 subjects for the polymorphism. Although DIA_3^1 frequency was about 0.8, a value similar to that found by others, Kopetz *et al.* (1979) apparently found 63 samples in their material which could not be typed. Oepen *et al.* (1980) found one DIA$_3$ 3-2 type, as well as 3 variants which had not previously been seen.

37.14 Phosphoglycolate Phosphatase

Phosphoglycolate phosphatase (PGP; 2-phosphoglycolate phosphohydrolase; E.C. 3.1.3.18) catalyzes the hydrolysis of 2-phosphoglycolate to yield orthophosphoric acid and glycolate. In 1978, Barker and Hopkinson found that human red cell PGP exhibited six electrophoretic phenotypes, which could be accounted for by three alleles operating at an autosomal locus, and called PGP^1, PGP^2 and PGP^3. The phenotypes were designated PGP 1, 2-1, 2, 3-1, 3-2 and 3. The PGP polymorphism was found to be expressed in a variety of tissues besides red cells, including leucocytes, cultured fibroblasts, muscle, intestine, kidney, spleen, testis, ovary, lung and brain. An electrophoretic system was described which was suitable for PGP typing, in that it was relatively specific for this phosphatase activity. The enzymes were most active with phosphoglycolic acid as substrate, of the 33 organic phosphates tested. Some activity was demonstrable with 3-phosphoglycerol. Family studies confirmed the postulated mode of inheritance, and the gene frequencies in a random sample of Europeans were: $PGP^1 = 0.826$, $PGP^2 = 0.129$, and $PGP^3 = 0.045$. As such, some 20% of Europeans are expected to be PGP 2-1, about 1% would be PGP 2 and about 7% would be PGP 3-1, the remainder of the phenotypes being relatively infrequent.

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Bibliographic Notes to References for Unit VI

§[1] *Forensic Serology News* [*Forensic Serol. News*] is an uncopyrighted newsletter publication for the informal exchange of information among forensic serologists in the U.S.A. It was published by the Criminal Justice Center, John Jay College of Criminal Justice, City University of New York from January 1975 through June 1976, at which time it was taken over by the Forensic Sciences Foundation, Inc., located in Rockville, MD until March, 1980, when it moved to Colorado Springs, CO. The newsletter was issued in six number per year from 1975 through 1977, and in four numbers per year thereafter. It has always been issued in one volume per year.

§[2] *Japanese Journal of Human Genetics* [*Jpn. J. Hum. Genet.*] has the Japanese title *Jinrui Idengaku Zasshi*

§[3] *Japanese Journal of Fertility and Sterility* [*Jpn. J. Fertil. Steril.*] has the Japanese title *Nippon Funin Gakkai Zasshi*

§[4] *Medical Journal of Osaka University* [*Med. J. Osaka Univ.*] has the Japanese title *Osaka Daigaku Igakubu*

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

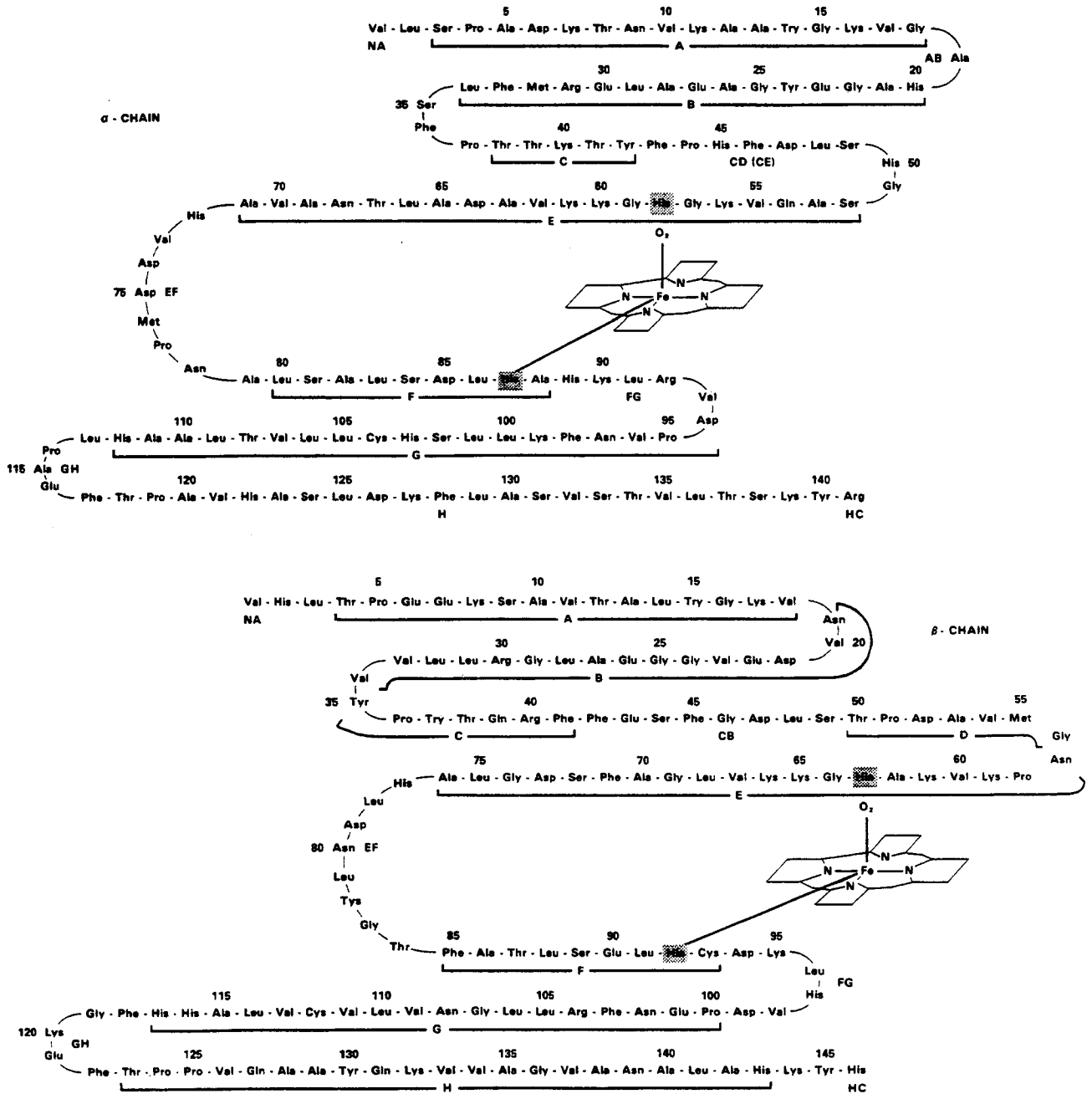

Figure 38.1 ***α*** **and** ***β*** **Chain Sequences in Hemoglobin.**

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

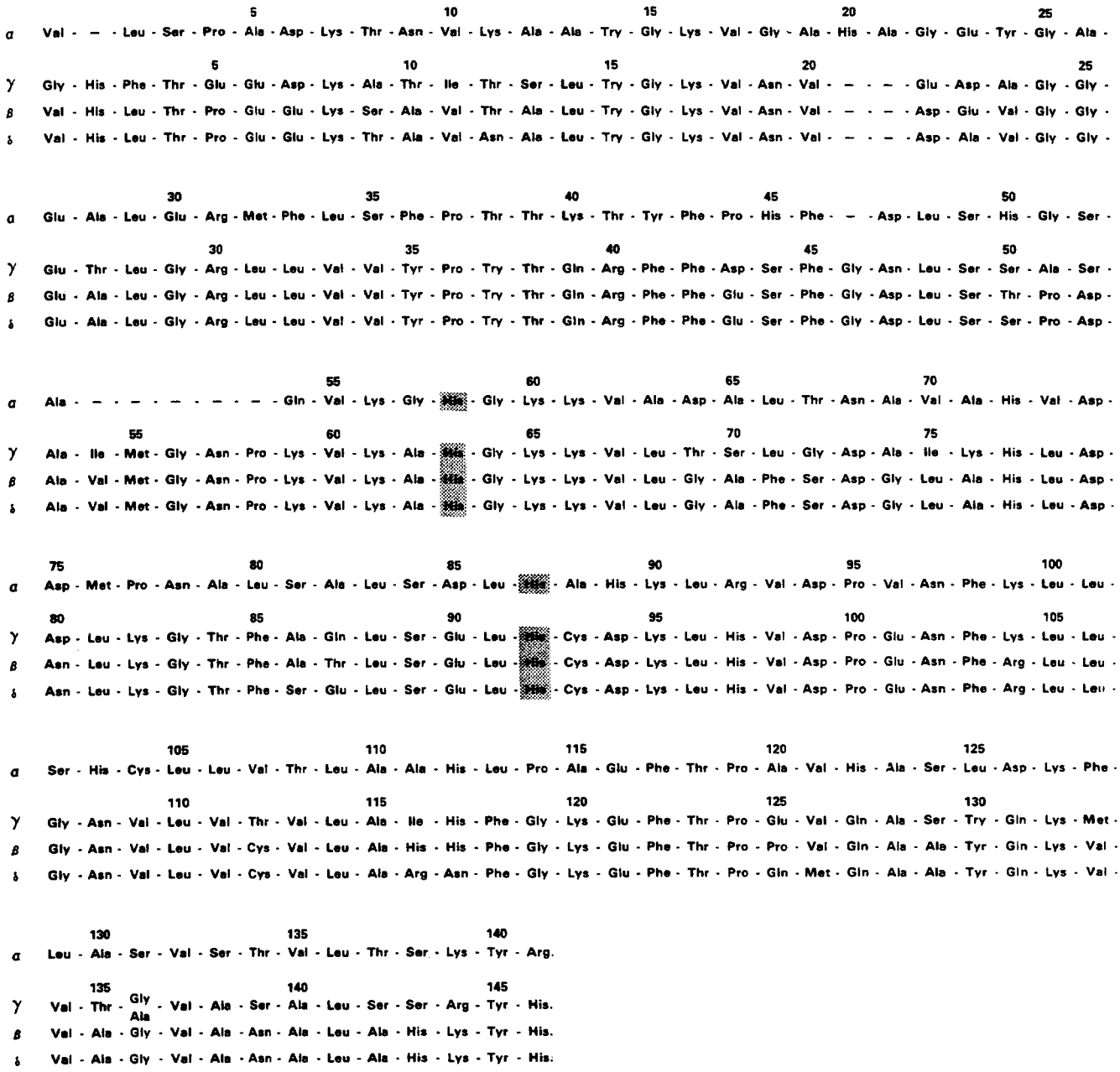

Figure 38.2 Sequences of the α, β, γ and δ Chains of Hemoglobin.

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

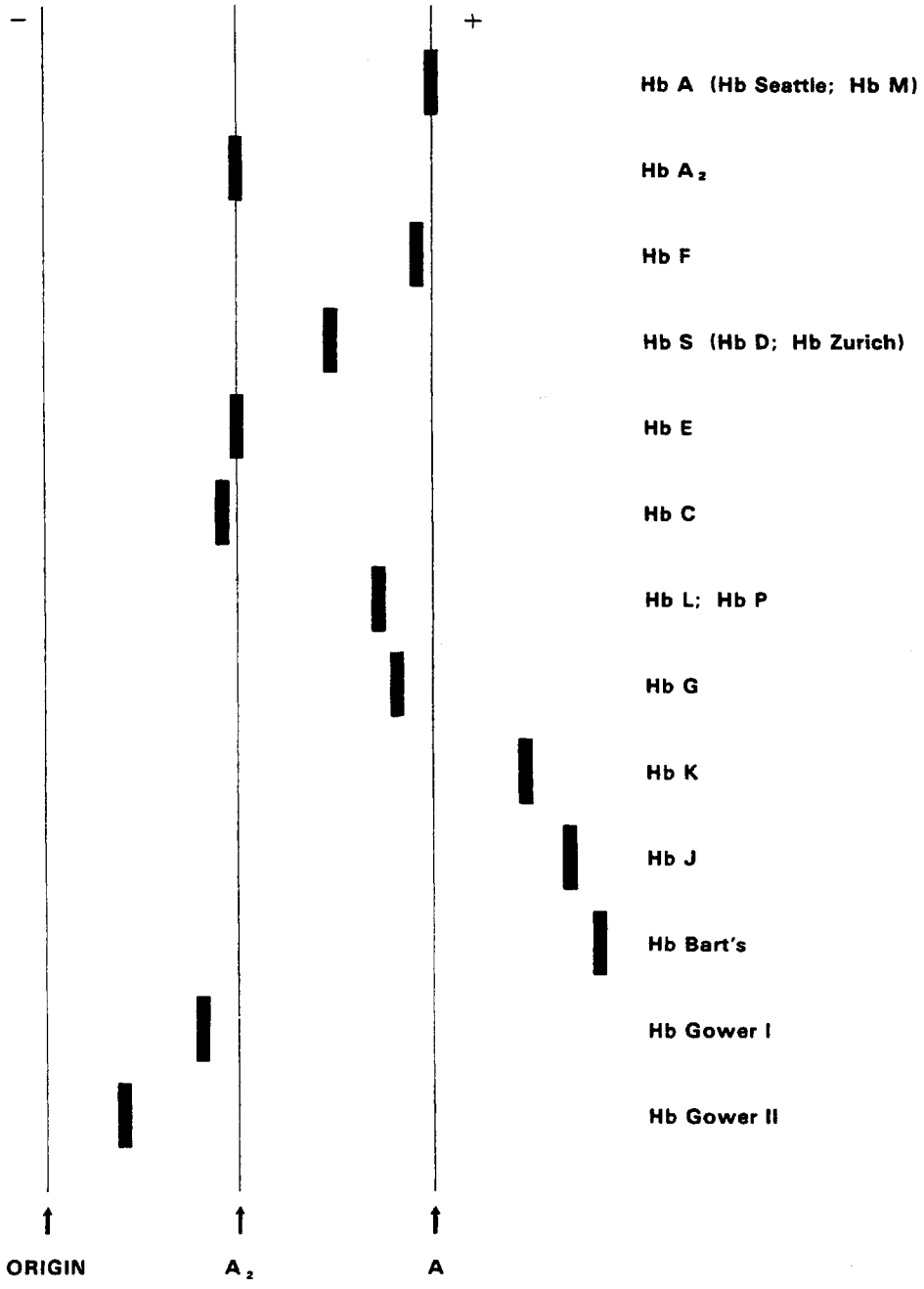

Figure 38.4 Relative Electrophoretic Mobilities of Some Hemoglobins at pH 8.6

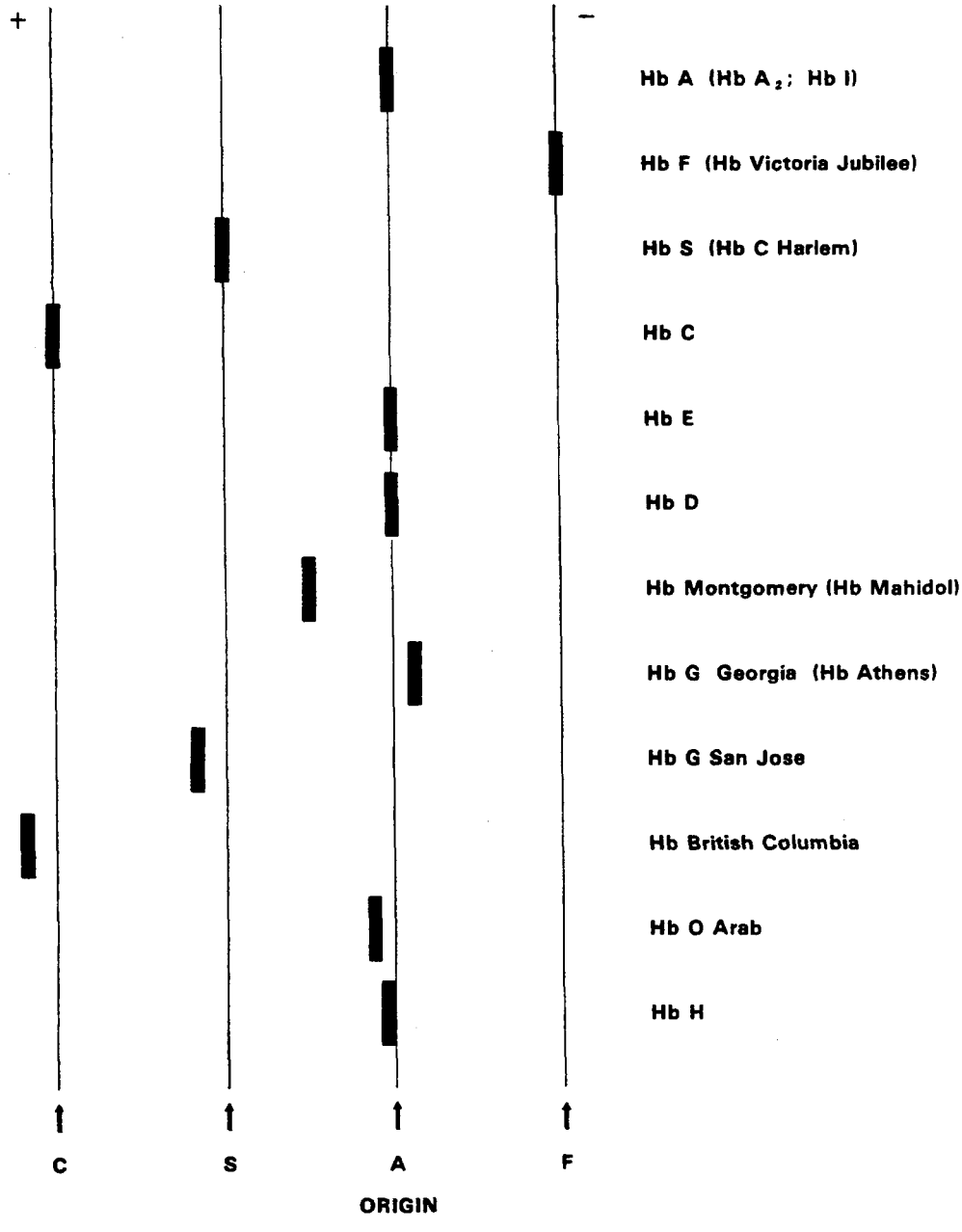

Figure 38.5 Relative Electrophoretic Mobilities of Some Hemoglobins of pH 6 in Agarose

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	600	600 (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, 1958
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.

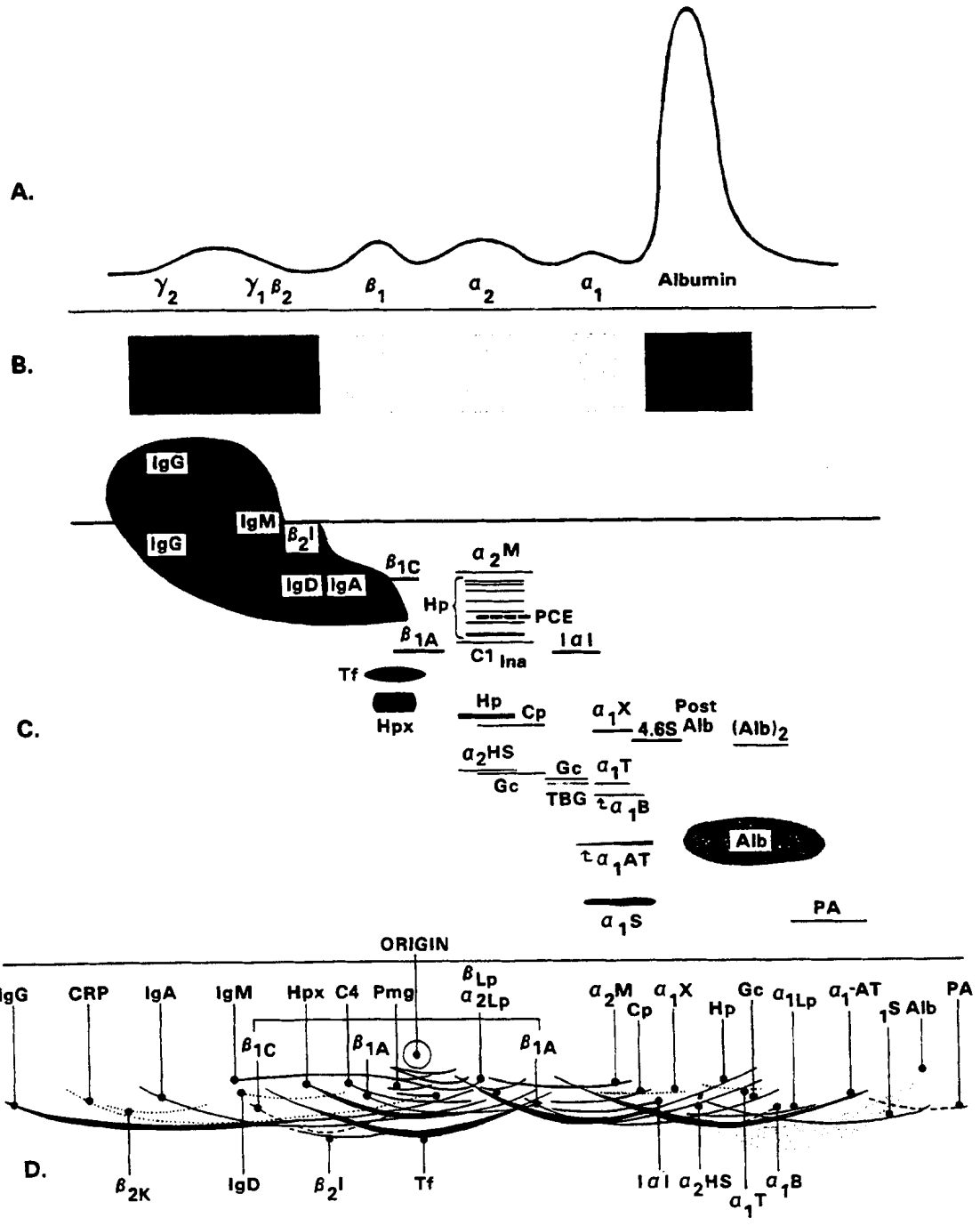

Figure 39.1 Plasma (Serum) Protein Profiles by Various Techniques

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,

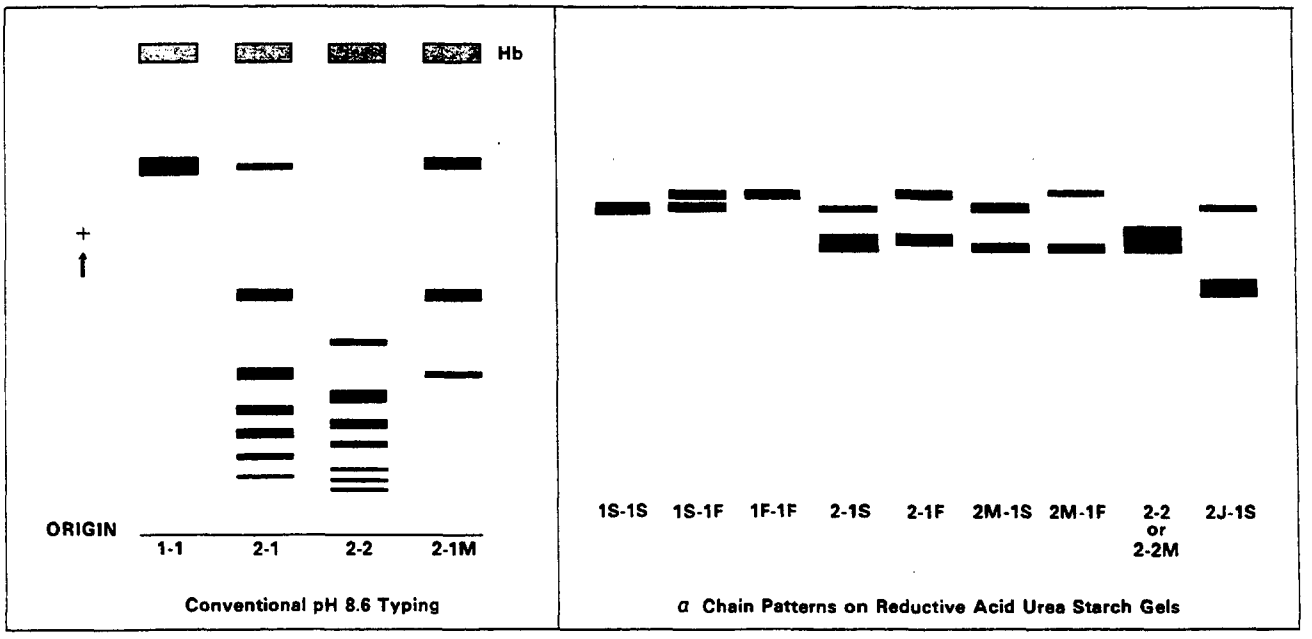

Figure 40.1 Electrophoretic Patterns of Hp Types and Subtypes

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,

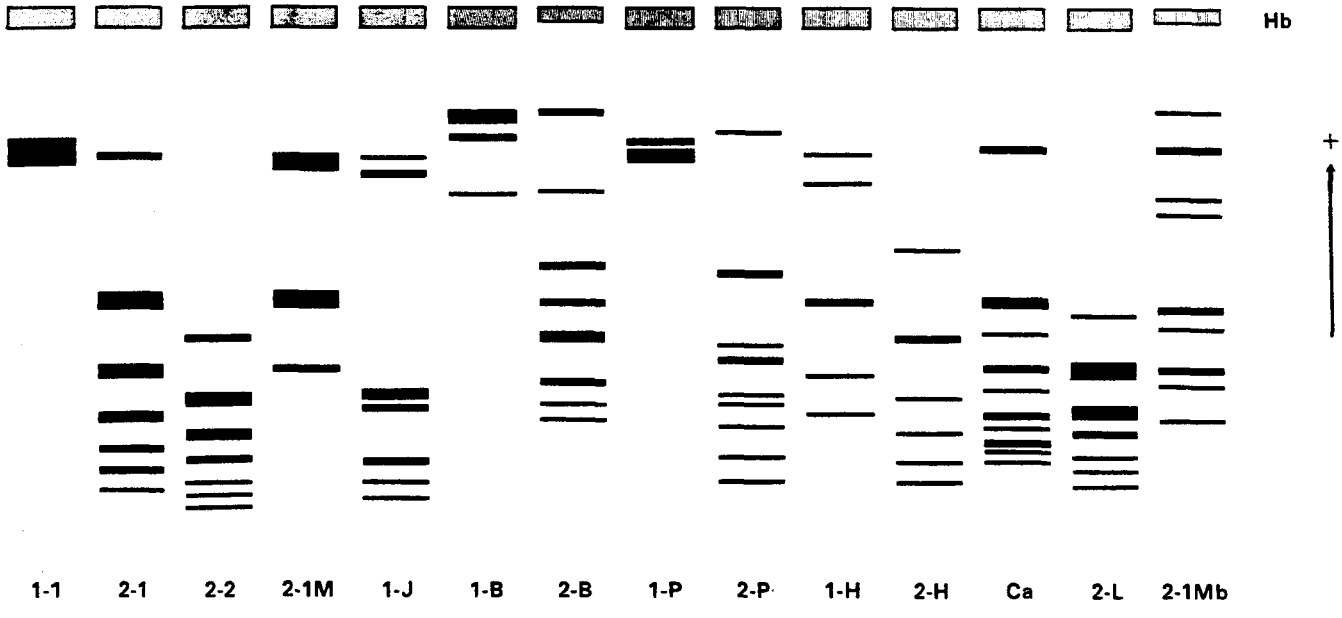

Figure 40.2 Electrophoretic Patterns of Some Haptoglobin Variants

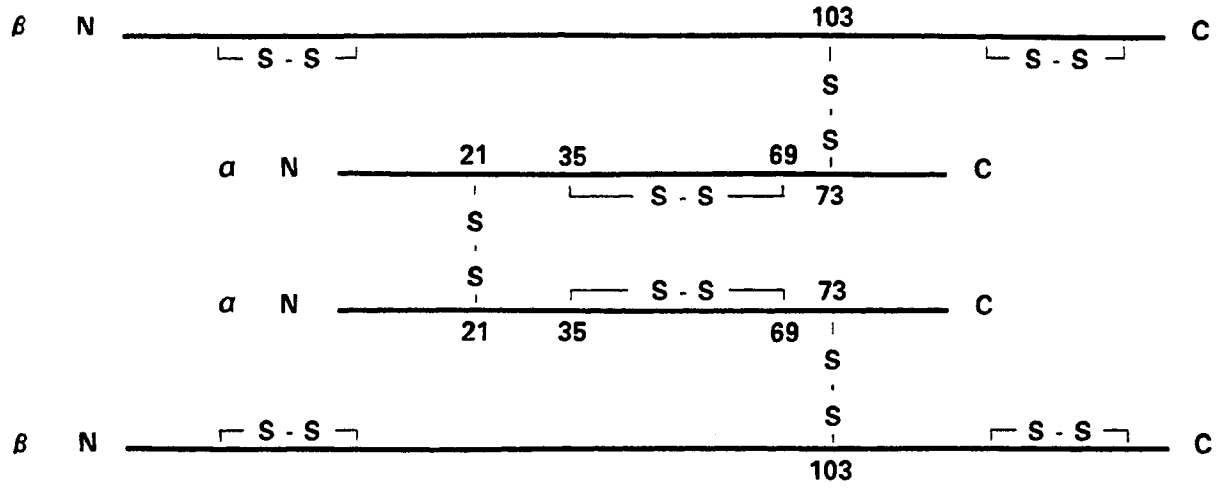

Figure 40.3 Diagrammatic Structure of Monomeric Haptoglobin

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