A CRITICAL REVIEW OF HUMAN HAEMOGLOBIN VARIANTS

PART II: INDIVIDUAL HAEMOGLOBINS

BY

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The authors believe that a reconsideration of some of the results of investigations so far carried out on individual haemoglobins would be salutary. A summary of previous work on their separation and characterization is therefore given, but without detailed discussion, since it is hoped that the general comments of Part I (Beaven and Gratzer, 1959) will suffice.

The possibility has been raised (Ager, Lehmann, and Vella, 1958b; Benzer et al., 1958) that samples of any given haemoglobin variant derived from different ethnic sources may be similar only in terms of particular properties, and that in reality many more haemoglobins than those enumerated below exist.

Haemoglobin A Components

Separation.—A minor component (A₂) has been separated from normal adult haemoglobin in starch block electrophoresis by Kunkel and co-workers (Kunkel and Wallenius, 1955; Kunkel and Bearn, 1957; Kunkel et al., 1957), Rosa (1958), Silvestroni, Bianco, and Muzzolini (1957), and by Bernasconi and co-workers from haemoglobin of normal adults and from subjects with pathological disorders (Bernasconi, 1956; Marinone and Bernasconi, 1956, 1958; Marinone et al., 1956), (Cabannes et al., 1957a, have found as much as 27% in one pathological case), by Gerald and Diamond (1958a), Masri et al. (1958), Josephson et al. (1958), and Ceppellini et al. (1958).

A variable minor component has been found by Berry and Chanutin (1957, 1958) using free boundary electrophoresis, and fast minor components have been found by the same technique by Hoch (1950) and by Shooter and Skinner (1956). Separation and estimation of minor components by paper electrophoresis have been achieved by Aksoy, Lehmann, and Eng (1957), Silvestroni and Bianco (1957), Modiano (1957), Hoch and Barr (1956), de Traverse et al. (1958), and by Cabannes et al. (1957a). Two minor components, one fast and one slow-running, have been obtained by Morrison and Cook (1955b, 1957) by ion exchange chromatography, and by the same technique Allen et al. (1958) found three fast-running components but no slow ones and Huisman et al. (1958) one, or possibly two, fast and one slow; three components in all were reported by Van Fossan (1954) on alumina columns (but see also Prins and Huisman, 1956a). Derrien and
Reynaud (1953) found six components by free boundary electrophoresis, but these results have been shown by Itano (1956) not to be valid (see also N.A.S.-N.R.C. Conference, 1958, p. 199). Separation of minor components in starch gel is reported by de Grouchy (1958) and Labie, Rosa, Dreyfus, and Schapira (1958). Some five components have been claimed by Derrien and co-workers from solubility measurements (Roche and Derrien, 1951, 1953; Derrien, 1957; Derrien in N.A.S.-N.R.C. Conference, 1958, p. 183), and two by Allison and Tombs (1957) (see Itano (1953a) and the discussion in Part I). Recently Masri et al. (1958) have obtained an “A4” component as well as A2 on starch, but since its proportion is so small that it can be detected only in samples of 500 to 1,000 mg. little can be said about its identity as a haemoglobin rather than a complex denaturation product (cf. also Ceppellini et al., 1958).

Alkali-resistant components have from time to time been claimed to be present in normal adult haemoglobin (Singer et al., 1951; Huisman et al., 1955a; Künzer, 1955), and for the most part these have been discussed by Betke et al. (1954), the present authors (v. Part I), and others. The suggestion by Roques and de Prailauné (1953) that 8–18% of normal adult haemoglobin is alkali resistant on the basis of denaturation following treatment with formaldehyde receives no support from other directions. The stepwise nature of the denaturation reaction after treatment with formaldehyde is described by Brada (1957).

Characterization.—A preparation of crystalline haemoglobin from Haemoglobin F has been reported by Morrison and Cook (1955a); this is said to be heterogeneous, there being one major component (90%) and three minor ones. In addition these authors claim that one of the minor components of haemoglobin isolated from their ion exchange columns gives a different haem from that of Hb-A. 

Morrison and Cook deduce that heterogeneity of normal adult haemoglobin arises partially from differences of the prosthetic group. Such a conclusion is not to be taken for granted, however, since, as already pointed out, minor components could well arise through partial denaturation, oxidation or binding, and the effect of such factors on the fission of the haem-globin linkage and the nature of the haem is unpredictable. Such a conclusion finds no support in the work of Kunkel and co-workers (Kunkel et al., 1957; Ceppellini, 1956), who have prepared the A2 fraction in bulk and have carried out much careful characterization. The homogeneity of the sample
et al., 1955e). Separation by ion exchange chromatography for diagnostic (Huisman and Prins, 1955, 1957; Prins and Huisman, 1955) and preparative purposes (Huisman and Prins, 1955; Morrison and Cook, 1955b, 1957; Cook and Morrison, 1956; Allen et al., 1958; Huisman et al., 1958; Saha, 1959) has been described but is believed by the present authors to be often open to criticism (v. Part I).

Characterization.—The presence of several components in foetal haemoglobin has been suggested: (i) On the basis of irregularities in the alkali denaturation curve (White, F. D., et al., 1950; Betke, 1951; Kleinkecht, 1953); the validity of this argument has been disproved by Rossi-Fanelli et al. (1955a), Kubowitz (1957), Betke (1952a) and others; (ii) on the basis of salting-out experiments by Roche, Derrien and co-workers (Roche and Derrien, 1951; Roche et al., 1953a, 1953b, etc.); (iii) by Van Fossan (1954) on the grounds of its behaviour on alumina columns (but v. Prins and Huisman, 1956b); and (iv) on ion exchange resin columns by Prins and Huisman (1956) (though not on the modified cellulose columns of Huisman et al., 1958) and more particularly by Allen et al. (1958). The latter authors report differences in the isoleucine contents of the fractions.

The alkali denaturation of Hb-F has been the subject of much study by Haurowitz, Hardin, and Dicks (1954), Betke (1953a), Itano (1957b), Kubowitz (1954, 1957), Golden and Layton (1956), Polosa and Motta (1955), White and Beaver (1954), Beaven et al. (1956), Singer et al. (1951), Jonxis and Huisman (1956), and others. The alkali denaturation of the carboxy form has also been studied by Derrien and Laurent (1955b), Putignano and Cognetti (1952), Beaven et al. (1958), Polosa, Motta, and Falsaperla (1957a), and Künzer (1953a, 1955). The last author (1953a) also studied the behaviour of cyammet-haemoglobin and methaemoglobin, and the denaturation of the reduced form has been commented on by Kubowitz (1957) and Betke (1951). The balance of evidence seems to indicate that the alkali denaturation reactions of the carboxy and met derivatives are not of practical value. The denaturation of Hb-F in erythrocytes in blood films has also been demonstrated (Kleihauer, Braun, and Betke, 1957). Acid denaturation has been studied by Putignano and co-workers (Putignano and Martino, 1951; Putignano and Cognetti, 1952), Penati et al. (1955c), Chini, Perosa, Putignano, and Bini (1954) and Kleihauer (1957). Foetal haemoglobin appears to be up to three times more resistant than adult, but the method is not sufficiently sensitive to be of analytical value (Penati et al., 1955c).

Differences between haemoglobins F and A have also been established by Gardikas et al. (1953) in relation to their rates of denaturation by urea and sodium salicylate, though White and Kerr (1957) record that differences in behaviour of Hb-F and Hb-A are trivial with urea, nicotinamide, guanidine, sodium benzoate, and sodium salicylate. Heat denaturation and coagulation of the two proteins have been compared by Betke (1953c) and by Betke and Greimacher (1954b).

The solubility curves of Hb-F have been examined by Derrien and co-workers with the results already mentioned, by Giraud, Orsini, and Le Pouillain (1956), Polosa et al. (1957c, 1957d), and Pagliardi et al. (1954), all using the salting-out procedure, and by Itano (1953a), White and Beaver (1954), Jope and O'Brien (1949), and Wyman, Rafferty, and Ingalls (1944). Laurent, Bouscayrol, Dunan, and Borgomano (1956) have carried out salting-out experiments using adult, foetal, and thalassaemic methaemoglobins, and observe differences in the shapes of the curves compared with the corresponding carboxy-haemoglobins.

The crystal habits of haemoglobins F and A are discussed by Jope and O'Brien (1949) and by Perutz et al. (1951).

The nature and significance of the oxygen dissociation curves of adult and foetal haemoglobins are discussed by Allen et al. (1953).

A number of specific chemical differences also exist, most of which have already been mentioned. In particular very exhaustive studies have been made of oxidation and reduction reactions in the haemoglobin-methaemoglobin system with a number of reagents and the results discussed in relation to oxygen dissociation behaviour. This work is due to Betke (1952b, 1953b, 1957), Künzer and co-workers have commented on the oxidation reaction with nitrates (Künzer et al., 1953), chlorates (Künzer and Saffer, 1954), and on spontaneous oxidation of solutions of Hb-A and Hb-F (Künzer and Künzer, 1952). This reaction has also been examined by Betke et al. (1956d).

No differences in the affinities of Hb-A and Hb-F for isocyanides were reported by Murayama (1955).

The characteristic feature of the Hb-F ultraviolet absorption spectrum is well known: the tryptophan band anomaly and its application to analytical work is described by Beaven and
Huisman (1952), Rich (1952), and Penati et al. (1954d). Intensity variations in the ultra-violet absorption spectra of mixtures of Hb-A and Hb-F have also been examined by Penati et al. (1955d). The infra-red spectrum has been studied by Greinacher et al. (1953), who find no significant differences between haemoglobins F and A, and by Penati et al. (1955b), who report small but consistent differences.

Andersch et al. (1944) reported differences in sedimentation behaviour, but this has not been confirmed (Taylor and Swarm, 1949) by diffusion studies.

Betke and Greinacher (1955) have recorded differences between the adsorption capacities of alumina for haemoglobins A and F, and Jonxis (1939) found small differences in the spreading rate in monolayers. Betke (1950) has discovered a difference also between the peroxidase activities of the two haemoglobins, the magnitude of which does not seem sufficient to permit of its analytical application by the benzidine colour reaction.

The immunological specificity of Hb-F has been established (Darrow et al., 1940; Vecchio and Barbagallo, 1950; Sansone and Durando, 1951; Chernoff, 1953b; Aksoy, 1955; Diacono and Castay, 1957). The number of titratable sulphydryl groups under various conditions has been reported by Murayama (1957a, 1957b, 1958) and Hommes et al. (1956); the latter authors have drawn conclusions as to the structure of the molecule (v. Part I). End group analyses have been carried out by Schapira and Dreyfus (1954), Masri and Singer (1955), Huisman and Drinkwaard (1955), Schroeder and Matsuda (1958), and Huisman and Dozy (1956). Complete amino-acid analyses are described by Rossi-Fanelli et al. (1954), Dustin et al. (1954), van der Schaaf and Huisman (1955a), Huisman et al. (1955b), and Stein et al. (1957); the globin alone has been examined by Dustin et al. (1954) and Perosa (1950). Dustin et al. (1954), Huisman et al. (1955b), and van der Linden (1950) discuss the isoleucine content, which is the greatest structural difference between Hb-F and other haemoglobins. The conclusions of Derrien and Laurent (1955a) as to the presence of Hb-F in normal adult blood appear to be doubtful (v. Part I). The amino-acid composition of the alkali-resistant fraction occurring in sickle-cell anaemia haemoglobin has been examined by Huisman et al. (1954).

There remains the perennial question whether Hb-F is identical with the alkali-resistant haemoglobin associated with thalassaemia. This has been the subject of prolonged controversy, but only a few of the most cogent recent arguments will be quoted. Briefly, the evidence for the separate identity of the alkali-resistant haemoglobin of thalassaemia is as follows: (i) Differences in free boundary electrophoresis patterns observed by Derrien and Reynaud (1955); these results, however, were obtained in cacodylate buffer under conditions already discussed, and the possible effects of differences in other cell constituents should also be considered. The Tiselius electrophoresis experiments recently described by Derrien (N.A.S.-N.R.C. Conference, 1958, p. 195) showed heterogeneity effects only after some 20 hours. As Itano (ibid. p. 199) points out, such a separation would correspond to minute charge differences such as could be brought about by a change in pK of one group. Moreover, in the course of excessively long runs artefacts arising from diffusion, electrolysis, and other effects are to be expected. (ii) Differences in the alkali denaturation rates of the carboxyhaemoglobins (Vecchio, 1946; Putignano and Cognetti, 1952), a technique which, as already stated, seems to be unreliable. (iii) Differences in the acid denaturation rates of the oxy and carboxy derivatives (Perosa and Bini, 1954; Chini et al., 1954); here again, as Penati et al. (1955c) point out, the method is too insensitive for any reliable conclusions to be drawn, (iv) A difference in immunological behaviour (Diacono, 1957), and (v) alleged small differences in the rates of migration on paper (Perosa and Bini, 1954); the imponderable factors involved in paper electrophoresis again make results of this kind open to criticism. All this evidence rests on the use of ambiguous techniques. Against this may be set the many properties of thalassaemia haemoglobin identical with those of Hb-F of cord blood. These are summarized by Liquori (1951), Liquori and Bertinotti (1951), Huisman et al. (1956), and de Marco and Trasarti (1957), and include crystal structure, alkali denaturation rates, solubility of the reduced, oxy- and carboxy haemoglobins, spectral features, i.e., position of the tryptophan band, electrophoretic and chromatographic behaviour, and amino-acid composition. The authors believe the identity to be established beyond reasonable doubt.

**Haemoglobin S**

Since the identification of Hb-S as a new molecular species by Pauling, Itano, Singer, and Wells in 1949, this haemoglobin has been more thoroughly studied than any of the subsequently discovered pathological variants.
The separation of Hb-S from Hb-A by paper electrophoresis is good (Bergren et al., 1954; Larson and Ranney, 1953; Reynaud, 1953; Spaet, 1953; Gatto and La Grutta, 1955b; Smith and Conley, 1953; etc.), and it is also clearly demarcated in agar (Robinson et al., 1957; Giri and Pillai, 1956), starch slab (Kunkel et al., 1957), starch gel (Owen and Got, 1957; de Grouchy, 1958), and by isoelectric line spectra (Tuttle, 1956). Its behaviour in free boundary electrophoresis is also distinctive (Itano and Neel, 1950; Wells and Itano, 1951; Sturgeon et al., 1952; Zinsser, 1952; Singer et al., 1954; Shooter and Skinner, 1955), and indeed the first fractionation was achieved by this method (Pauling et al., 1949). The mobility variations observed by Anderson and Griffiths (1954), and ascribed to complex formation, have been considered in Part I. The behaviour of Hb-S on ion exchange columns appears also to be unambiguous (Morrison and Cook, 1955b, 1957; Huisman and Prins, 1955, 1957), and separation by paper chromatography has been claimed by Benhamou and Pugliese (1958).

Roche et al. (1952a) have attempted to show by salting-out curves that Hb-S is heterogeneous, but the criticisms already mentioned of these methods apply here. Goldberg (1956) found a double peak in the zone associated with Hb-S in paper electrophoresis, but no heterogeneity has been found by other methods. Havinga and Itano (1953) have shown that the globin of Hb-S is homogeneous.

In most respects the differences between Hb-S and Hb-A are small. Betke and Greinacher (1955) have found that the rate of oxidation with various reagents is in both cases identical, that heat denaturation and coagulation occurs somewhat faster in Hb-S, and that there are slight differences in adsorption affinity on alumina. Although the oxygen dissociation curve of blood from subjects suffering from Hb-S diseases is abnormal, this appears to be due to the presence of dialysable constituents, and the inherent oxygen affinity seems to be normal (Wyman and Allen, 1951; Becklake et al., 1955). Attempts have been made to establish immunological specificity, but these have met with some difficulty (Goodman and Campbell, 1953; Diacono and Castay, 1956a, 1956b). Recently, alleged spectral anomalies have been reported (McCord and Gadsden, 1958).

Differences in the number of titratable sulphhydril groups have been found by Ingbar and Kass (1951), Murayama (1956, 1957a, 1957c) and Hommes et al. (1956). Terminal residues have been estimated by Huisman and Drinkwaard (1955), Masri and Singer (1955), Huisman and Dozy (1956) and Havinga (1953). Amino-acid analyses by Schroeder et al. (1950), Huisman et al. (1955b), and Stein et al. (1957) have shown no significant differences from Hb-A, but a difference in the composition of one peptide from partial hydrolysis was established by Ingram (Ingram, 1957, 1958; Hunt and Ingram, 1958b) by his finger-printing technique. Hunt and Ingram (1958a) find the trypsin-resistant portions of Hb-S and Hb-A to be identical. The "differential titration" method of Scheinberg et al. (1954, N.A.S.-N.R.C. Conference, 1958, p. 277) has been applied to Hb-S (v. Part I). In addition Havinga (1953) demonstrated the presence of small amounts of phosphorus in this protein. He has also noted that the fission of the haemoglobin linkage is more difficult than in Hb-A. The globins from bloods containing Hb-S have also been prepared and examined by Havinga and Itano (1953).

The most interesting and distinctive property of Hb-S, and the one from which its physiological manifestations arise, is its low solubility in the reduced state. This difference does not extend to the oxy form (Dervichian et al., 1952b; Itano, 1953a), or to the methaemoglobin (Perutz and Mitchison, 1950), but in the reduced state the solubility is such that tactoids are formed in solution (Harris, 1950). The striking optical properties of sickled cells (Perutz and Mitchison, 1950) are attributed to intra-erythrocytic crystallization of the Hb-S. The behaviour of reduced Hb-S and its crystallography have been studied by Perutz et al. (1951), and the viscosity changes which accompany deoxygenation, and which are pH-dependent, are described by Greenberg, Kass, and Castle (1957) and by Griggs and Harris (1956). The effect on the solubility of other haemoglobinins which may be present is important (Singer and Fisher, 1953; Singer and Singer, 1953). The viscosity of reduced haemoglobinins in solution has been investigated by Allison (1957), who refers to mutual interactions and discusses structural implications. Oxyhaemoglobinins A and S behave identically in respect of viscosity (Nakamura, 1955). The relatively high oxygen tension at which cells containing a mixture of Hb-S and Hb-C sickle is of interest (Allison, 1956). The same author has also made a study of the rate of sickling in relation to the proportion of Hb-S present. It is found that the latter governs the rate of sickling directly. The high osmotic and mechanical fragility of cells containing Hb-S (Lange et al., 1951) arises
presumably from the crystallization of the haemoglobin in the reduced state, though it is likely that the nature of the stroma is also relevant. It may be noted that Dervichian et al. (1952a, 1952b) and Ponder (1951) take a rather different view of the sickling phenomenon, believing that the birefringence is the consequence of an "ultrastructure" formed by the interaction of haemoglobin with lipoproteins of the cell walls. This theory is in some measure confirmed by the electron microscopic studies of Bessis, Nomarski, Thiéry, and Breton-Gorius (1958). Perosa, Ramunni, Birri, and Manganelli (1957) report the appearance of crystals in red cells by phase contrast microscopy only when Hb-A is present as well as Hb-S.

**Haemoglobin C**

Hb-C separates from Hb-A in paper electrophoresis as shown by Bergren et al. (1954), Spaet (1953), Smith and Conley (1953), Schneider (1954), Ranney et al. (1953), etc. It has also been separated on agar (Robinson et al., 1957), by isoelectric line spectra (Tuttle, 1956), and by free boundary electrophoresis (Ranney et al., 1953; Schneider, 1954; Singer et al., 1954, etc.). It moves more slowly than Hb-A on ion exchange columns (Morrisson and Cook, 1955b, 1957; Huisman and Prins, 1955, 1957) and is said to separate in paper chromatography (Benhamou and Pugliesi, 1958).

Ranney et al. (1954) found the sedimentation rate to be similar to that of Hb-A, and Betke (1957) stated that its rate of oxidation with potassium ferricyanide also shows no difference. Independent solubility measurements have been carried out but do not agree. Huisman et al. (1955c) report that the solubility of carboxyhaemoglobin C in phosphate buffers at pH 6.5 is lower than that of carboxyhaemoglobins S, A, and F.

On the other hand, Derrien et al. (1955a) find the solubility under very similar conditions to be high (the latter authors also find three Hb-C components). Again Itano (1953a) and Thomas, Motulsky, and Walters (1955) report the solubility of reduced Hb-C to be higher than Hb-A, whereas Huisman et al. (1955c) give the solubility as being slightly lower. The low solubility would be consistent with the formation of intra-erythrocytic crystals in blood containing Hb-C (Diggs, Kraus, Morrison, and Rudnicki, 1954), and the crystallization of the haemoglobin in citrated blood in vitro (Kraus and Diggs, 1956). Itano et al. (1956) suggest that these conflicting results may be explained by the existence of two different haemoglobins of identical mobility.

The number of titratable sulphhydril groups is found to be the same in Hb-C as in Hb-A (Murayama, 1957a, b, 1958; Hommes et al., 1956). Terminal residues have been determined by Huisman and Drinkwaard (1955), Masri and Singer (1955), and by Huisman and Dozy (1956) with carboxypeptidase. Amino-acid analyses have been carried out by Huisman et al. (1955b, 1955c) and by Stein et al. (1957), and a difference in the composition of a peptide unit has been discovered by Hunt and Ingram (1958b). The "differential titration" method of Scheinberg et al. (1954) has also been applied to Hb-C, but these authors' conclusions as to the number of free carboxyl groups are inconsistent with the amino-acid results.

An increased fragility in cells containing Hb-C has been reported (Spaet et al., 1953).

**Haemoglobin D**

Hb-D can be separated from Hb-A by paper electrophoresis as shown by Itano (1951), Bergren et al. (1954), Polosa and Motta (1957), Chernoff (1958), etc. Both on paper and in free boundary electrophoresis (Itano, 1951) as well as starch gel (de Grouchy, 1958) it is indistinguishable from Hb-S but is reported to run in the Hb-A position in agar electrophoresis (Robinson et al., 1957). According to Huisman and Prins (1957) it is also identical with Hb-S in its behaviour on ion exchange columns. Itano (1951) and Derrien, Cabannes, Laurent, and Roche (1955b) distinguished between haemoglobins S and D by differences in solubility: whereas the solubility of the carboxyhaemoglobins is similar, in the reduced form Hb-D has a solubility which is an order of magnitude greater than that of Hb-S. Derrien et al. (1955b) again find at least two components to be associated with Hb-D. Immunologically Hb-D is found to be indistinguishable from Hb-A (Chernoff, 1958). As already mentioned, Benzer et al. (1958) found differences in the amino-acid composition of samples of Hb-D from three different sources.

**Haemoglobin E**

Hb-E migrates on paper at the same rate as A₂. Cabannes et al. (1957a) claim, however, to distinguish between them at pH 6.5, though such conditions are unfavourable for paper electrophoresis (Itano et al., 1956). Separations of Hb-E from Hb-A on paper are given by
Chernoff et al. (1954), Itano et al. (1954), Vella (1957), and, in an extensive survey of populations in which the variant occurs, by Brumpt, de Traverse, Brumpt, and Coquelet (1957a and b) and Brumpt, Brumpt, Coquelet, and de Traverse (1958). Itano et al. (1954) also separated and isolated Hb-E from the ascending limb of a free boundary electrophoresis apparatus, and separation in starch gel has been described (de Grouchy, 1958). Its chromatographic behaviour is described by Prins and Huisman (1956b; Huisman and Prins, 1957) and Morrison and Cook (1955b, 1957). The solubility of Hb-E is similar to that of Hb-A (Itano, 1953b; Sturgeon, Itano, and Bergren, 1955).

Huisman and Drinkwaard (1955) give the number of valine residues per molecule of Hb-E as 5, and Jonxis, van der Schaaf, and Prins (1956) find that its amino-acid composition is identical with that of Hb-A. Ingram (cited in Ager, Lehmann, and Vandepitte, 1958a) has found that, although haemoglobins E and A2 appear indistinguishable by all physical and chemical criteria so far applied, there are differences between these proteins in terms of amino-acid sequences.

Increased erythrocyte fragility has been reported to be associated with the presence of Hb-E (Chernoff et al., 1954). The haemoglobin is also precipitated with brilliant cresyl blue or sodium metabisulphite (Vella, 1957), and inclusion bodies have been observed in a case of Hb-E/thalassaemia (Minnich, NaNakorn, Tuchinda, Wasi, and Moore, 1956).

**Haemoglobin G**

The behaviour of Hb-G in paper electrophoresis is described by Chernoff (1955), Schwartz and Spaet (1955), Schwartz, Spaet, Zuelzer, Neel, Robinson, and Kaufman (1957), Edington and Lehmann (1954), Edington et al. (1955), and Ager et al. (1958a). A comparison with Hb-D is given by Cabannes, Raffi, Boineau, Domenech, and Guivarc'h (1958). Resolution has also been claimed by free boundary electrophoresis (Edington et al., 1955), and this variant is said to migrate between haemoglobins A and E on ion exchange columns (Ager and Lehmann, 1957). The solubility of reduced Hb-G is found by Edington et al. (1955) and Edington and Lehmann (1954) to be much higher than that of Hb-S and slightly lower than that of Hb-A. Crystals of Hb-G and mixed crystals of haemoglobins A and G have been prepared but not examined crystallographically (Edington et al., 1955).

**Haemoglobin H**

This "fast" haemoglobin and its separation by paper electrophoresis has been described by Rigas et al. (1955, 1956). It has also been examined by free boundary electrophoresis (Rigas et al., 1955, 1956; Thorup, Itano, Wheby, and Leavell, 1956), by starch slab electrophoresis (Hedenberg, Müller-Eberhard, Sjölin, and Wranne, 1958), and by ion exchange chromatography (Ager and Lehmann, 1958). Its isoelectric point under specified conditions is given by Rigas et al. (1956) as 5.6 as against 7.05 for Hb-A. It is less soluble than Hb-A in the reduced form (Rigas et al., 1955; Vella, 1957). It is evidently unstable and is readily precipitated irreversibly from its solutions and denatured by freezing (Rigas et al., 1955). The rate of this process increases with decreasing pH or oxygen pressure (Rigas et al., 1956; Vella, 1957). The formation of inclusion bodies is also associated with this haemoglobin (Gouttas, Fessas, Tsverenis, and Xefteri, 1955; Vella, 1957). Its presence in erythrocytes is associated with elevated fragility (Vella, 1957; Brain and Vella, 1958). The amino-acid composition is said by Huisman (1958) to differ appreciably from Hb-A.

**Haemoglobin I**

The separation of a haemoglobin designated Hb-I is described by Rucknagel, Page, and Jensen (1955) and by Cabannes et al. (1957b). It has been examined by free boundary and paper electrophoresis (Rucknagel et al., 1955; Ager et al., 1958a) and ion exchange chromatography (Huisman and Prins, 1957), and its sedimentation behaviour, oxygen dissociation, and solubility are reported (Rucknagel et al., 1955) to resemble those of Hb-A.

**Haemoglobin J**

This was discovered by Thorup et al. (1956), and its migration on paper and in Tiselius electrophoresis is described by these authors. Patterns on paper are also shown by Ager and Lehmann (1958), and its behaviour in starch gel is described by de Grouchy (1958). Its chromatographic properties have been investigated by Huisman and Prins (1957) and Huisman, Noordhoek, and da Costa (1957). Thorup et al. (1956) report that its solubility in the reduced state is substantially greater than that of Hb-A. Huisman (1958) gives the amino-acid composition of Hb-J, which resembles that of Hb-A fairly closely.
**Haemoglobin K**

This designation was first applied to a fraction separated by Battle and Lewis (1954) by free boundary electrophoresis, no clear result being obtained on paper. Itano et al. (1956) were unable to obtain any such indications of a new species with the same sample, though Hb-F appeared to be present. The designation has since been applied to the so-called Liberia II haemoglobin found by Robinson et al. (1956), Vella (1958), and Trincão, Almeida Franco, Gomes, and Ferreira (1958), as well as apparently by Cabannes et al. (1957b) and Cabannes and Buhr (1955). The migration on a column is said to be slightly faster than Hb-A (Ager and Lehmann, 1958). No characterization studies appear to have been carried out. Robinson et al. (1956) comment that electrophoretic examination was impeded, because, when the haemoglobin solution was diluted with cacodylate buffer, a precipitate appeared.

**Haemoglobin L**

This was reported by Ager and Lehmann (1957) and migrates more slowly than Hb-A in paper and free boundary electrophoresis (Ager et al., 1958a; Ager and Lehmann, 1957) and on columns (Ager and Lehmann, 1957, 1958). Its solubility is said to be similar to that of Hb-A (Ager and Lehmann, 1957). The amino-acid composition as given by Huisman (1958) is somewhat similar to Hb-A.

**Haemoglobin M**

This was obtained as the methaemoglobin by Hörlein and Weber (1948, 1951), and was characterized by these authors, as well as by Kiese et al. (1956) and Heck and Wolf (1958), in terms of its spectrum and that of its derivatives, oxygen binding, oxidation-reduction kinetics, and the abnormality of its globin. Gerald et al. (1957) believe that they have isolated the precursor haemoglobin by electrophoresis on starch block, and Pisciotta et al. (1957) describe a separation on paper. Rossi-Fanelli et al. (1957) also describe an abnormal methaemoglobin, associated with an anomalous crystal form as well as an abnormal spectrum. A second Hb-M variant has recently been described (Gerald, 1958; Gerald and George, 1959).

**Haemoglobin N**

This is the Liberia I haemoglobin reported by Robinson et al. (1956). Its migration on paper (Robinson et al., 1956; Ager and Lehmann, 1958; Trincão, Almeida Franco, and Nogueira, 1959) and on columns (Ager and Lehmann, 1958) has been described. There are no reports of characterization studies.

**Haemoglobin O**

This is the Buginese X variant reported by Eng (1957). The evidence for its existence rests solely on its electrophoretic and chromatographic behaviour (Eng, 1957; Eng and Sadono, 1958).

**Haemoglobin P**

A pre-foetal haemoglobin (Allison, 1955) has been claimed to exist by Künzer (1957b), Künzer and Drescher (1956; Drescher and Künzer, 1953, 1954), and Halbrecht and Klibanski (1956; Halbrecht et al., 1958), all of whom claim to have found it in young foetuses. On the other hand Walker and Turnbull (1955) have found only Hb-F in similar foetuses, and Beaven et al. (1951) likewise found no evidence for any additional component in Tiselius electrophoresis under conditions giving good resolution of Hb-A and Hb-F. Halbrecht and Klibanski claim that the migration of this pigment in paper electrophoresis is significantly slower than that of haemoglobins F and A, though unresolved, and that it is alkali-resistant. Künzer (1957b) and Künzer and Drescher (1956) find that its alkali resistance is intermediate between that of haemoglobins A and F, and that the tryptophan band is in the position characteristic of Hb-F.

**Haemoglobin Q**

This is yet another haemoglobin which is known only in terms of its migration velocity, which is said to be identical with that of Hb-L on paper and agar but differing from it in its mobility on ion exchange columns (Vella et al., 1958). Its presence has been claimed only in association with Hb-H.

**Galveston Haemoglobin**

The evidence for the existence of this variant (Schneider and Haggard, 1957, 1958) rests entirely on its migration velocity in paper and free boundary electrophoresis; in the first it behaves similarly to Hb-L, and in the second it is unresolved, except on addition of further Hb-A. On ion exchange columns its migration is given as slightly slower than Hb-A, but it is not resolved and no separation occurs in electrophoresis on agar gel (Schneider and Haggard, 1958).

**Bart's Haemoglobin**

This was reported by Ager and Lehmann (1958) to be the fastest haemoglobin yet discovered, both on paper electrophoresis and ion exchange columns. Its alkali resistance is said to be intermediate between haemoglobins A and F and its tryptophan band is nearer the foetal than the
adult position. It was found in a child in the presence of Hb-F.

South Vietnam Haemoglobin

Recently Albahary, Dreyfus, Labie, Schapira, and Tram (1958) reported a new haemoglobin in South Vietnamese. This was said to migrate on paper at pH 8.6 with a velocity intermediate between Hb-F and Hb-A. No separation from Hb-A, however, was observed, but the above authors claimed that the diffuseness of the zone indicated the presence of a mixture of Hb-A and the new variant. Examination of samples containing the alleged variant by starch gel electrophoresis led de Grouchy et al. (1958) to announce the existence of no less than three South Vietnam haemoglobins, two slightly slower and one slightly faster than Hb-A and none of them resolved.

Hopkins I and Hopkins II

Smith and Torbert (1958) have reported two electrophoretically new haemoglobins. No physical or chemical characterization is given.

Norfolk Haemoglobin

Ager et al. (1958b) have described a variant occurring in an English family, with an electrophoretic mobility very slightly different from Hb-A but separating from it on ion exchange columns.

Lepore Haemoglobin

This has been reported by Gerald and Diamond (1958b) as separating in the same position as Hb-S or Hb-D on starch block. The samples show no trace of abnormal haemoglobin, however, when subjected to free boundary and agar gel electrophoresis or ion exchange chromatography. Solubility measurements on the recovered pigment have been carried out.

Other Abnormal Haemoglobins

An abnormal fast haemoglobin was discovered in a child by Fessas and Papaspyrou (1957). It was not alkali-resistant, and its possible identity as Hb-I, J, or K was ruled out by genetic considerations (v. supra). This haemoglobin disappeared almost completely some three months after birth (cf. Hb-F).

A haemoglobin said to migrate on paper in a similar position to haemoglobins E and C and associated with thalassaemia in a Frenchman has also recently been mentioned (André, Bessis, Dreyfus, Jacob, and Malassenet, 1958).

Chernoff (N.A.S.-N.R.C. Conference, 1958, p. 179) also mentions a "Durham I" haemoglobin, but gives no description.

Conclusions and Practical Implications

It will be clear that the recognition of a new haemoglobin variant—and indeed in many cases the identification of an abnormal pigment with one of the hitherto accepted variants—is a matter demanding considerable caution. Workers in the field have of late recognized this fact and have exercised more restraint than previously.

Apart from the difficulties and consequent ambiguities arising out of the inherent chemistry of the haemoglobin molecule, a situation has now been reached where all the known haemoglobin variants (and nearly 30 have been named) lie within a quite narrow range of electrophoretic (and chromatographic) mobility. In the present state of protein chemistry and the almost complete absence of specific differences in chemical reactivity (apart from alkali denaturation rate) between the haemoglobin variants, electrophoresis and/or chromatography must remain the basic methods for distinguishing between such closely related molecular species. In addition, it is necessary to appreciate that these techniques have limitations, both of resolving power and reproducibility. This is particularly true of paper electrophoresis, which, having a number of obvious virtues for routine purposes, remains the most widely applied technique. It will be seen from the foregoing summary of the individual haemoglobin variants that the evidence for the existence of a number of these pigments is based largely on paper electrophoresis, in which they are said to migrate "between haemoglobins A and F." It is evident that here the limitations of the technique are being disregarded.

The same strictures apply in greater or lesser degree to the other methods available, but it seems likely that electrophoresis in agar gel and possibly in starch gel (bearing in mind the striking results which this technique has yielded for serum proteins) may with careful development give substantially greater resolution of haemoglobins. It is already clear that agar is particularly valuable in situations involving Hb-F, especially in the absence of specialized spectrographic facilities; this method should not be neglected.

Chromatography on ion exchange columns is clearly subject to the same limitations as zone electrophoresis, possibly in greater degree, since the migration rates cannot be related, even as a first approximation, to charge differences. It would appear that the fundamental principles
controlling such separations of large molecules have not yet been fully elucidated.

The recognition of a given haemoglobin variant in zone electrophoresis is rendered much more accessible with the commoner variants, but may not be feasible with rare or less well-established variants that have been reported on only a few occasions. The potential value of a readily accessible reference collection of haemoglobin variants is obvious, but the operation of such a scheme would encounter serious problems in the collection, identification, storage, and distribution of large numbers of samples of valuable and unstable material.

The only technique which can, in principle, characterize a haemoglobin variant in reproducible, quantitative terms is Tiselius free-boundary electrophoresis, from which a curve relating absolute mobility to pH under given conditions can be derived, even in the absence of reference samples. From the published data it is not yet certain whether, in fact, such measurements could be made with sufficient precision for this purpose. In view of possible complications arising from interactions in solution between haemoglobin species, it may be necessary to envisage the prior fractionation of mixtures, so that only single-component systems are characterized in this way. It is clear that such studies are not feasible for the routine examination of large numbers of samples.

As already suggested (Annotation, British Medical Journal, 1958, vol. 1, p. 1469) the final recognition of a haemoglobin variant as a unique molecular species may have to await the perfection of methods for the complete amino-acid analysis and determination of the residue sequence and interchain linkages. The work of Ingram and his colleagues on the separation and identification of peptides derived from haemoglobins by enzymatic hydrolysis is a notable advance along these lines.

Addendum

In a subject expanding as rapidly as the one under review, the rate at which new material accumulates defeats any attempts at completeness from the outset. The recent publications mentioned below either bear on the arguments outlined in Part I (Beaven and Gratzer, 1959) or indicate the trends of current work on the haemoglobin variants.

Much recent work has been concerned with the refinements of protein fractionation techniques as applied to haemoglobin. These include starch gel electrophoresis, which has been applied by Labie et al. (1958) to the evaluation of Hb-A2, and the examination of a number of abnormal haemoglobins in a "discontinuous" system, in which one buffer is used in the preparation of the gel and another for the electrophoresis (McGrouchy, 1958); improved separation by this means has been claimed.

The use of agar at high pH (in the region of 8.6) has been developed and applied successfully, mainly to animal haemoglobins (Fine, Uriel, and Faure, 1956; Monnier and Fischer, 1958). Whether these procedures have advantages over the low-pH method of Robinson et al. (1957) is questionable; certainly no comparable resolution of Hb-F is observed.

Good results in the separation of haemoglobins A, S, C, and F have been claimed by an improved paper chromatography technique (Benhamou and Pugliese, 1958).

The salting-out curves of normal adult haemoglobin have been re-examined by Polosa et al. (1958a) and an analysis of accuracy and reproducibility is given.

A discussion of the globin structure in relation to the reversible dissociation of haemoglobins A, S, and C (see Part I, p. 13) based on ultracentrifuge and electrophoretic studies is given by Itano and Singer (1958). The most important result of this detailed work is confirmation that hybrid molecules, arising from dissociation and recombination of two different molecular species, are not formed.

A significant contribution to the investigation of the cysteine content of normal adult haemoglobin in the native and partially denatured states has been made by Cole, Stein, and Moore (1958). The results enumerated in Part I (p. 12), notably those of Allison and Cecil (1958), may be re-examined in the light of this work.

A good deal of attention has been focused lately on the non-haem proteins which have at various times been demonstrated in haemolysates (see Part I, p. 15 seq.). Two such proteins were observed by Polosa, Motta, and Pennisi (1958c), and the same authors (1958d) isolated the more abundant of them and examined its absorption spectrum, which was found to show only a protein band. This protein is presumably to be identified with the "X" component reported by Derrien et al. (1956), which is absent in the foetus and appears 11 months after birth. Laurent, Depieds, and Derrien (1958) present evidence, based on alkali denaturation and immunological properties
of the "X" protein, in comparison with those of serum proteins of comparable mobility, that this substance is stromal or intra-erythrocytic in origin.* The isolation of the protein by electrophoresis, and the presence of up to three other non-haem proteins, is described by Derrien, Laurent, and Reynaud (1958). The most interesting characteristic of this protein (Laurent, Borgomano, and Derrien, 1958) is its high isoleucine content—stated by these authors to be some six times greater than that of Hb-A and twice that of Hb-F. (But note that Stein et al. (1957) give the isoleucine content of electrophoretically purified Hb-A as less than 0.02 g./100 g. compared with about 1.45 for Hb-F, a ratio of greater than 1/70.) The protein moreover remains even after recrystallization of the haemoglobin. These observations may render suspect the characterization of fractionated haemoglobin zones in terms of isoleucine content (Allen et al., 1958) and bear also on the discussion of effects which could arise from the binding of haemoglobin with extraneous material (Part I). One other recent publication should be mentioned in the same connexion. Anderson and Turner (1959) find that 3% of the total haemoglobin content of the erythrocyte remains integrally bound with the stroma, being inseparable by repeated washing; or, conversely, that over 50% of the total stromal material is haemoglobin. This is judged to be in the native state because drastic treatment is avoided in the preparation of the red cell "ghosts," and also because of the colour changes which accompany removal and readmition of oxygen. It is to be supposed that any stromal material which is not precipitated and remains in solution also contains bound haemoglobin, and might be expected to have a characteristic behaviour of its own in fractionation experiments.

Two reports have appeared of a new foetal haemoglobin variant. Fessas, Mastrokalos, and Fostiopeoulos (1959) describe an "Alexandria" haemoglobin, present in an infant to the extent of 18.3% at birth, falling to 2.2% after 15 weeks. This pigment is said to migrate more slowly than Hb-S at pH 8.6 in paper and starch block electrophoresis, and similarly to Hb-S at pH 6.7. It is not observed in agar at pH 6.5 and resembles Hb-F in respect of alkali denaturation and position of the tryptophan fine-structure band. Vella, Ager, and Lehmann (1959) claimed to have observed a similar substance in a cord blood specimen of Chinese origin. Migration at pH 8.6 on paper was similar to Hb-S, and ion exchange chromatography and agar gel electrophoresis revealed only the zones associated with Hb-F and Hb-A. No difference from Hb-F was observed in alkali denaturation or position of the tryptophan band, but the spectra shown are not adequate to support this conclusion. Another haemoglobin variant has been claimed to be present in an infant by Bernard, Schapira, Dreyfus, Mathé, Rosa, and Labie et Cohen (1958). This is said to have mobility intermediate between haemoglobins A and F in paper at pH 8.6 and to run slightly more slowly than Hb-A on starch slab and slightly faster on an ion exchange column.

Further evidence has been claimed for the existence of an embryonic haemoglobin (Halbrecht, Klibanski, and Bar Ilan, 1959). It is unresolved on paper, but is said to show up on ion exchange columns, though it is not clearly visible in the published figure (no reason is given why it should not be identified with the small component frequently observed by Prins and Huisman (1956b) in cord blood under the same conditions). It is also reported to be present in cases of full-term infants with certain congenital disorders, which are supposed to be associated with the delayed disappearance of this pigment.

*It may be noted, however, that Fine et al. (1956) regard small components detected in agar electrophoresis as serum constituents which remain in spite of repeated washing of the erythrocytes.

References
