A CRITICAL REVIEW OF HUMAN HAEMOGLOBIN VARIANTS

PART I: METHODS FOR SEPARATION AND CHARACTERIZATION

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At the time of writing, the existence of some 29 haemoglobins, both normal and pathological, seems to have received widespread acknowledgment. A number of excellent reviews (Allison, 1958; Betke, 1956, 1958; Itano, 1956; Itano, Bergren, and Sturgeon, 1956; Malassenet, 1957; Itano, 1957a; Huisman, 1958) have appeared in recent years describing the incidence, nature, and clinical and genetic manifestations of these haemoglobins, but making for the most part little mention of their characterization or of the available criteria for heterogeneity, on which the evidence for the existence of many of the haemoglobin variants rests. It is felt that a critical examination of these underlying factors together with an attempt to evaluate the results achieved by the various techniques which have been exploited is timely. In particular attention will be focused on a number of factors

arising out of the inherent nature of haemoglobin which may be expected to have a fundamental bearing on the interpretation of experimental results.

Mention must first be made of the techniques that have been applied to the comparative study of human haemoglobin variants.

METHODS IN THE STUDY OF HUMAN HAEMOGLOBINS

Zone Electrophoresis

Paper.—This method has had the most widespread application on account of its cheapness and simplicity. It should be noted that the evidence for a considerable number of abnormal haemoglobins is based solely on the results of paper electrophoresis experiments. It is not proposed to discuss the method here, since reviews of general techniques are available

(Bergren, Sturgeon, and Itano, 1954; White, Beaven, and Ellis, 1956; Goldberg, 1957), but mention should be made of one or two of its limitations. In the first place the behaviour of a protein on paper depends on a number of incalculable factors, in particular electro-osmotic and adsorption effects, and it is not generally possible to obtain absolute mobility measurements by this means, though the measurement of relative mobilities by comparing three strips carrying the sample, an Hb-A (adult haemoglobin) control, and a mixture of the two, run simultaneously, has been proposed by Toporek (1956). Irreversible adsorption can be largely eliminated by the addition of a fast-running, non-reacting protein to the buffer mixture (Hoch and Barr, 1956). The method remains an essentially comparative one, though here again attempts have been made to incorporate mobility markers into the system (Goldberg, 1957) or to run markers by way of control (de Wael and Punt, 1957). Heating effects are of importance and can give rise to spurious heterogeneity, and Topalović-Avramov, Draškoci, Kapetanović, and Lero (1956) have observed that substantial changes in the rate of migration of one serum protein component relative to another, amounting in extreme cases to inversion of position, is brought about by varying the distance between the point of application of the sample and the anode. Reference must here be made to a cautionary article by Moinat and Tuller (1957), who suggest that paper electrophoresis experiments be carried out in the cold room. The present authors believe that the results of paper electrophoresis have in many cases been insufficiently scrutinized, and a good example exists of the pitfalls into which the method can lead, in which the observations of an allegedly new haemoglobin reported from one laboratory (Battle and Lewis, 1954) could not be repeated with the same sample under rigorous conditions (Itano *et al.*, 1956).

Typical results obtained by paper electrophoresis are given by Motulsky, Paul, and Durrum (1954), Chernoff (1955), Smith and Conley (1953), Larson and Ranney (1953), Reynaud (1953), and other workers, and a comparison of results in different buffers is given by Morrison, Rudnicki, and Digs (1954). The separation of Hb-A and Hb-F (foetal haemoglobin) has been observed on paper only by Gatto and La Grutta (1955a) and by Haynie, Dobson, and Hettig (1957) in cases where Hb-F is present to the extent of 40% or more. A good estimate of lower Hb-F concentrations has been claimed by the former authors in terms of rate of migration of the compound zone compared with that of pure Hb-A.

Quantitative work by dye-elution or staining and subsequent photometry has been carried out (Larson and Ranney, 1953; Ranney, Larson, and McCormack, 1953; Modiano, 1957; Silvestroni and Bianco, 1957), though it is conceivable that the results are in some cases rendered ambiguous by the non-specificity of the stains which have been used (Modiano, 1957; de Traverse, Coquelet, and Brumpt,

1958). Other limitations on the accuracy of such work (Shooter, Skinner, and White, 1958) exist (*v. infra*).

Paper electrophoresis is ill adapted for preparative work, though attempts at elution have been made (White *et al.*, 1956). Scheinberg, Harris, and Spitzer (1954) have described a "differential titration" method on paper, which sets out to obtain information on the number of charged groups in different haemoglobin variants. The conclusions (see also Scheinberg in N.A.S.-N.R.C. Conference, 1958, p. 277) are, however, seen to rest on experiments carried out at extreme pH values, at which the haemoglobins are extensively denatured, and are accordingly of limited validity.

Recently good results have been obtained by the use of cellulose acetate in place of paper (Kohn, 1957).

Starch Gel.—This method was devised by Smithies (1955) and has been successfully applied to human haemoglobins by Owen and Got (1957), who have achieved the difficult Hb-A/Hb-F separation in a striking manner, and also by de Grouchy, Schapira, and Dreyfus (1958). The method has preparative possibilities since the haemoglobin can be extracted from the segregated zones by electrophoresis (Moretti, Boussier, and Jayle, 1958) or by freezing (in the latter case at the expense of some denaturation) (Smithies, 1955).

Starch Block (Kunkel, 1954).—The electrophoresis of haemoglobin in beds of starch grains was described by Kunkel and Wallenius (1955) and has been used by Kunkel and his co-workers for the isolation of the A₂ component of adult blood (Kunkel and Bearn, 1957; Kunkel, Ceppellini, Müller-Eberhard, and Wolf, 1957).^{*} The method lends itself particularly well to preparative work since the pigment is easily extracted, and Kunkel and Bearn (1957) have run 20 ml. samples of 8% haemoglobin solutions on large starch blocks. The technique has been used for routine analysis by Bernasconi (1956), Marinone, Bernasconi, Morsiani, and Lucci (1956), Marinone and Bernasconi (1957), and by Gerald and Diamond (1958a), who applied their samples in the form of cyanmethaemoglobin, a derivative whose use is rendered particularly desirable in virtue of its relatively great stability. Recently Masri, Josephson, and Singer (1958), making use of electro-osmotic flow on starch block, have obtained three minor haemoglobins as well as some non-haemoglobin fractions from normal adult haemolysates (see also Ceppellini, Kunkel, and Dunn, 1958). A remarkable separation of haemoglobins F and A has very recently been reported by Künzer and Ambs (1958) by the use of very high voltages on starch slab. This may well be a major advance in the problem of haemoglobin fractionation.

Agar Gel.—Electrophoretic separation of haemoglobins in agar gel was described by Giri and Pillai

^{*}Kunkel *et al.* (1957) have also used polyvinyl chloride as supporting medium in much the same way.

(1956), who were able to distinguish between haemoglobins A and S at pH 8.6. Giri, Rao, and Natarajan (1958) also demonstrated the presence of minor protein components in human red cell haemolysates by the same means. Robinson, Robson, Harrison, and Zuelzer (1957) devised a method of separating haemoglobins at low pH, and have obtained good results, notably in the resolution of Hb-F.

It should be mentioned that the order of migration of the haemoglobins on agar (Robinson *et al.*, 1957) differs in some instances from that obtaining in other media. It is to be surmised that migration of haemoglobins on agar involves adsorption or molecular-sieve effects. Disagreeable artifacts can occur, apparently by overloading (Robinson *et al.*, 1957). A method of electrophoresis in a liquid film containing a small quantity of agar has been described (Ressler, Nelson, and Oster, 1958), and has been applied to haemoglobins by Zak and co-workers (Zak and Jarkowski, 1958; Zak and Sun, 1958). A micro-electrophoresis method on agar has also been reported (Monnier and Fischer, 1957, 1958).

Continuous Electrophoresis.—A number of methods of continuous electrophoresis for preparative use have been devised (Brattsten, 1955), and one such has been applied successfully to the fractionation of turtle haemoglobins by Ramirez and Dessauer (1957b).

Free Boundary Electrophoresis

This technique has found wide application in the study of haemoglobin (Zinsser, 1952; Itano *et al.*, 1956; van der Schaaf and Huisman, 1955b, etc.) though the results have not been unequivocal. It has the particular merit of yielding mobility values for each species under given conditions, and from these the iso-electric points can be interpolated. Large samples are required, and though the method was used for the isolation of Hb-E (Itano *et al.*, 1954) its preparative use is small. A detailed theoretical discussion of the free boundary technique and its limitations, together with its specific application to the fractionation of Hb-A and Hb-F, is given by Hoch (1950), and Shooter and Skinner (1955) discuss the optimal conditions for resolution of Hb-S (sickle-cell haemoglobin). The drastic dependence of the results obtained for proteins on the selection of a suitable buffer is discussed by Woods (1958).

Micro-electrophoresis

The micro-electrophoresis technique of Antweiler has proved inadequate for the study of such closely related species as the human haemoglobin variants (Waris, 1954).

Iso-electric Line Spectra

The interesting iso-electric line spectra of Kolin (1955) have been obtained with human haemoglobins by Tuttle (1956). This is evidently a method of

considerable potentialities, in virtue of its simplicity and its fundamental nature, since separation depends solely on the iso-electric points of the species present.

Chromatography

Paper.—The paper chromatography of haemoglobins is of questionable value (Vella and Lugg, 1957). In all work of this type solvents have been used which do violence to the haemoglobin molecule, and the results would seem to defy interpretation (Fiori, 1957; Sansone, 1952; Sansone and Cusmano, 1950; Sansone, Cusmano, and Ravazzoni, 1951; Andersch, 1953), though consistent diagnostic results have been claimed (Berlingozzi, Rapi, and Dettori, 1953; Rapi, Dettori, and Salvioli, 1956; Illari and Marengi, 1955; Penati, Lovisetto, Turco, and Abrate, 1954a). Penati, Lovisetto, Turco, and Hutter (1954b) state that they can distinguish clearly between samples of haemoglobin from normal and thalassaemic subjects by the shape of the pigment smear in two-dimensional paper chromatography, using 50% pyridine as eluent in both directions. Goldberg (1958a) has investigated the electrophoresis of haemoglobins on paper impregnated with a micro-powder of "amberlite IRA-400," a strongly basic ion-exchange resin, and here again the results are questionable, two, and sometimes three, major components being obtained.

A method has also recently been described (Jain, 1958) for separating haemoglobin components on paper with a superimposed pH gradient. Successive precipitation is said to occur and three bands have been obtained from Hb-A. The significance of the separation is as yet in doubt.

Starch and Alumina Columns.—An exhaustive study of the behaviour of adult and foetal haemoglobins on starch columns has been made by Penati and co-workers (Penati, Turco, Hutter, and Lovisetto, 1955e), who conclude that no useful results are likely to accrue.

Alumina columns have given rise to debatable results in the hands of Kruh (1952) and of Van Fossan (1954) (*v. infra*). Prins and Huisman (1956a) found that the apparent heterogeneity of haemoglobin samples on alumina columns varied with experimental conditions, and discarded this technique.

Ion-exchange Columns.—Ion-exchange columns were first used for the fractionation of haemoglobins by Boardman and Partridge (1954, 1955), who separated mixtures of animal haemoglobins under carefully controlled conditions on the cation-exchange resin "amberlite IRC-50." (It may be mentioned that Boman (1955) has shown that elution of haemoglobin from an anion-exchange column under mild conditions is possible, but no use appears as yet to have been made of this observation.) No adequate separation of human Hb-A and Hb-F mixtures has been attained under the conditions of Boardman and Partridge (Beaven and Gratzner, 1958). Their procedure was modified by Morrison and Cook (1955b, 1957), Cook and Morrison (1956), and Allen,

Schroeder, and Balog (1958) and used to resolve minor components in normal adult blood, and also by Huisman and co-workers (Huisman and Prins, 1955, 1957; Prins and Huisman, 1955). The latter workers have developed two techniques: first, elution of haemoglobins from columns involving step-wise changes of buffer, a procedure which invites criticism on the grounds that spurious peaks can be generated by the assimilation of the tail of a zone by the new buffer, and secondly, the development of zones in a flat cuvette under conditions where the R_f value of the protein is too low to permit of elution at a useful concentration. It may be pointed out that the procedures described by Huisman and Prins (1955) imply incomplete equilibration of the resin with the buffer, and also depend on non-equilibration of the protein sample, since dialysis against the buffer to be used is specifically avoided. Whilst not calling into question the diagnostic value of Huisman's cuvettes, the authors have found that fractionation of Hb-F and Hb-A under these conditions is partly illusory. Mixtures of equal parts of Hb-F and Hb-A in the carboxy form were applied to columns under the condition specified by Huisman and Prins, and examination of the emergent zones spectrographically showed that separation of the two proteins was incomplete. The same was found to be true under cuvette conditions, when the separate pigment zones were extracted and analysed.

Huisman (1957b) gives figures for the percentage of Hb-F in mixtures applied to columns and the percentages of Hb-F and Hb-A in the zones ascribed to these components. These reveal that in general, and especially where the proportion of Hb-F is large, the zone identified with Hb-F contains up to some 16% more haemoglobin than the total Hb-F applied in the mixture. If Huisman's photometric methods of estimation are correct, imperfect fractionation is clearly indicated. This may be identifiable with similar effects observed in electrophoresis (Shooter *et al.*, 1958) (*v. infra*).

It may be anticipated (*v. infra*) that the separation of Hb-A and Hb-F may cause more difficulty than that of most other pairs of human haemoglobin variants, but the need for a greater degree of scepticism in the interpretation of chromatographic phenomena is indicated. The work of Morrison and Cook (1955b) on the separation of minor components from normal adult haemoglobin is also evidently not unambiguous.

Allen *et al.* (1958), using phosphate buffers differing slightly in composition, obtain three minor components from Hb-A and at least one from Hb-F. These components are obtained by separation first with one buffer, followed by fractionation of one of the zones with another buffer. The results are not consistent with those of Morrison and Cook (1955b, 1957), both in the absence of any slow-running component whatever and in the proportions of minor components involved, but can be reconciled in part with the appearance of small, fast-running components from Hb-A and Hb-F on the columns of

Prins and Huisman (1956b). Allen *et al.* suggest that the difference in isoleucine content between the various zones, as revealed by analysis, is an indication of the validity of their results. On the other hand, since it has been shown that variations in isoleucine content can arise from impurities (Stein, Kunkel, Cole, Spackman, and Moore, 1957), the possibility must be borne in mind that the fractionation may arise from binding of haemoglobin with extraneous substances with or without a large isoleucine content. The alleged substantial difference between the isoleucine contents of oxy- and of carboxyhaemoglobin A (Derrien and Laurent, 1955a; Allen *et al.*, 1958), whether crystallized or not, also renders such an explanation possible.

The authors have examined the behaviour of haemoglobins on modified cellulose columns. These materials developed by Peterson and Sober (1956) have certain advantages over resin, in particular their large capacity. Partial fractionation of Hb-F/Hb-A mixtures has been achieved, but a number of remarkable artefacts have also been observed, where two well-defined zones appeared immediately on application of buffer and were found to have identical compositions. Huisman has reported successful fractionation on columns of modified cellulose (Huisman, 1957a; Huisman, Martis, and Dozy, 1958).

Solubility

The solubility of a haemoglobin, both in the oxy- or carboxy as well as in the reduced form, constitutes a valuable parameter for its characterization and at times identification (a notable example is the use of this method for distinguishing Hb-D from Hb-S). Moreover, the appearance of the solubility curve of a protein has assumed classical status as a criterion for heterogeneity. The application of this method to the human haemoglobins is discussed in detail by Itano (1953a, 1957a). On the other hand, it seems clear that unwarranted conclusions have often been drawn from the results owing to a failure to appreciate the limits of the accuracy of which such work is capable.

Two procedures are available, the variable solute and variable solvent (salting-out) methods. It must first be said that many haemoglobin mixtures give rise to solid solutions (Itano, 1953a, 1957a), and in such a case the concentration of protein in solution when plotted against the total concentration in the solid phase will give a smooth curve. The smaller the number of readings available the more likely is it that a false impression of heterogeneity will be gained. (If in fact all successive points are joined by straight lines, the plot will indicate the presence of as many components as there are points.) In the absence of solid solutions the detection of discontinuities in the solubility curve is still somewhat subjective. Goldberg (1958a) has carried out an

analysis of experimental precision in the measurement of the solubility of reduced haemoglobin in a phosphate buffer under fixed conditions. Samples tested with 10 preparations of the same buffer gave results varying within limits of 2% and duplicate determinations on 35 samples of Hb-A gave solubility variations of up to 14%, with a standard deviation of 3.22. (It was also noted that lower solubilities were obtained with samples which had been kept at ordinary temperatures for any length of time.) An examination of most published work on solubility curves, most of all that of Derrien and co-workers (*v. infra*), shows that the evidence for the presence of minor components in the protein is frequently based on deviations from the curve lying well outside these accuracy limits.

An example of the application of the variable solute technique to normal adult haemoglobin is given by Allison and Tombs (1957). These authors find that three components are present in the system, but Itano (1953a) is of the opinion that the method is insufficiently sensitive to detect the minor components revealed by electrophoresis (Kunkel and Wallenius, 1955; Kunkel *et al.*, 1957; etc.) and ion-exchange chromatography (Morrison and Cook, 1955b, 1957; Allen *et al.*, 1958) and present to the extent of only a few per cent. He finds that, within the limits of his own experimental accuracy, normal adult haemoglobin is shown to be homogeneous.

The same strictures must be said to apply to the salting-out (variable solvent) procedure. Certainly the minor components of normal adult blood, as demonstrated by other methods, could scarcely explain the proliferation of haemoglobins in the systems examined by Roche, Derrien, and co-workers (Roche and Derrien, 1951; Roche, Derrien, and Roques, 1952b, 1953a; Roche, Derrien, Gallais, and Roques, 1952a; Roche, Derrien, Diacono, and Roques, 1953b; Roche, Derrien, Reynaud, Laurent, and Roques, 1954). A number of explanations for the appearance of misleading discontinuities suggest themselves. Itano (1956) believes that changes in aggregation of the haemoglobin molecules due to interaction with smaller molecules or with ions may be responsible. Evidence of such changes under the influence of ions has been adduced on the basis of light-scattering experiments, but results of different workers conflict (Benhamou and Weill, 1956, 1957; Putzseys, Schonne, and Sempoux, 1955). More significant are the observations of Karvonen and Leppänen (1952a) working with sheep haemoglobins. These authors established a clear-cut correlation between the sodium ion concentration within the parent erythrocyte and the crystal habit of the haemoglobin. It was found that where the sodium ion concentration was low, precipitation of needles occurred at pH 7, whereas in other samples precipitates of plates were formed at pH values below 6. The solubility of the samples was found also to increase with the total haemoglobin concentration. Such effects, as Karvonen and Lepänen point out,

could arise not only with a protein mixture but also from the presence of intra-erythrocytic impurities in the solution. In a further study (1952b) these authors observed that the tendency of the haemoglobin to remain in a supersaturated form, when not even the appearance of the first crystals brought about an immediate "autocatalytic" precipitation, was substantially reduced if it had previously been precipitated in amorphous form. This is ascribed to the presence of impurities, which act as protective colloids, and remain in the mother liquor after precipitation. Such manifestations are clearly not to be ignored.

Another suggestion which has been made (Itano, 1953a, 1957a) to explain the superabundance of reported discontinuities in the salting-out curves is the occurrence of phase transitions at the high salt concentrations at which the experiments have to be carried out. A phenomenon of this kind was observed by Ogston and Tombs (1956) to occur in β -lactoglobulin, and they have proposed a simple criterion for ascertaining whether breaks in the solubility curve arise from true heterogeneity or from a phase transition. In the former case, the position of the break in the curve will depend on the initial protein concentration, whereas in the latter this will have no effect. It follows also that if the break is indeed due to a phase transition a minimal initial protein concentration must exist which will give a curve without breaks. This behaviour has not yet been reported for haemoglobins, but the A, F, and S variants are known to crystallize in more than one system (Perutz, Liquori, and Eirich, 1951; Jope and O'Brien, 1949). The onset of such phase transitions under experimental conditions must be regarded as a possibility; on the other hand it seems by no means unlikely that a given crystal form will persist in metastable state over a considerable range of conditions.

The authors believe, however, that a thorough scrutiny of the experimental parameters underlying the variable solute results of Derrien and co-workers is particularly desirable before any conclusions are drawn from them. It is hard to believe that experimental error might not absorb a number of the subtler deviations observed in the salting-out curve, which are greatly magnified in the differential graphs which are drawn on the basis of the changes in slope. (It may be noted that Polosa, Motta, and Lo Turco (1958) find that the magnitudes of the fractions obtained in this way from the haemoglobins of various animals vary with the individual.) In the first place, it is quite clear that in many cases the deviations of the points which define a discontinuity are not consistent with the limits of precision of solubility measurements set out by Goldberg (1958a) (*v. supra*). Secondly, the breaks, in the flatter parts of the curve particularly, depend on extinction measurements of accuracy lying very close to the limits for the standard type of spectrophotometer. Moreover, these depend again on the exact adherence to Beer's law of the dilution of aliquots over a wide range. The latter difficulties are circumvented by Pagliardi, Vitelli, and Gaidano (1954), who measure

protein nitrogen content rather than spectrophotometric extinction and obtain rather more convincing results, only small minor components of relatively low solubility, possibly representing impurities, being obtained from normal adult, cord blood, and thalassaemia haemoglobins. The reproducibility of the results of Derrien and co-workers may be explained at least in part by the fact that solute change increments are taken at the same points in all their experiments. Where a single point defines a discontinuity a peak or at least an inflexion appears on the differential plot.

It is clear that before any conclusions on the heterogeneity of haemoglobin solutions can be drawn from solubility curves a statistical analysis of the limits of experimental accuracy and precision is required, as well as a more detailed physico-chemical examination of the system, both in respect of the solution and of the solid phase.

Chemical Behaviour

Alkali Denaturation.—The alkali denaturation of haemoglobins has been the subject of detailed study. For diagnostic purposes, that is to say, for the estimation of Hb-F in samples, a number of efficient routine procedures are available (Ponder and Levine, 1949; Singer, Chernoff, and Singer, 1951; Beaven and White, 1953; Derrien and Laurent, 1954; Chernoff, 1955).

In general, alkali denaturation experiments must be carried out with oxyhaemoglobin. Repeated attempts have been made to carry out measurements of the alkali denaturation rates of the carboxy and cyanmet derivatives, and Künzer (1953a, 1957a), using the precipitation method, has claimed that the results of such experiments are satisfactory for the estimation of Hb-F, and, indeed, that the use of cyanmethaemoglobin is preferable to that of oxyhaemoglobin. His success has not, however, been confirmed in other laboratories (Derrien, Laurent, and Roche, 1953; Beaven, Ellis, and White, 1958; Polosa, Motta, and Falsaperla, 1957). Künzer has suggested elsewhere (Künzer, 1955) that inconsistencies in results obtained with cyanmethaemoglobin can arise from contamination with and binding of plasma proteins. It seems likely that carboxyhaemoglobin must be converted to the oxy form before denaturation can proceed, and that the variations in denaturation rates from sample to sample depend on the amounts of excess carbon monoxide present (Introna, 1952; Derrien and Laurent, 1955b). Furthermore, Kubowitz in a comprehensive paper (1957) has shown that an intermediate of the denaturation of carboxyhaemoglobin is a carboxy pigment. Complications might also arise from the stepwise degradation of the carboxy derivative. Itano has shown that the four haems combine successively with carbon monoxide, and that three intermediates between haemoglobin and carboxyhaemoglobin can be isolated electrophoretically (Itano and Robinson, 1957).

The mechanism of the reaction remains obscure, though Kubowitz has shown that denaturation occurs by a number of contiguous or simultaneous processes, and that it is not justifiable to assume a true first-order mechanism, a fact which has also been stressed by Künzer (1953b) and by Rossi-Fanelli, Azzone, and Mondovi (1955a), who found that the values of the apparent first-order constants, and the proportions of the components deduced from them varied substantially with the pH. If such an assumption is in fact made, it could follow that changes in slope in the denaturation rate curve are due to heterogeneity of the sample, the overall reaction rate being compounded of the first-order rates associated with each component. This error has led to claims for the existence of three foetal and varying numbers of adult haemoglobins (Jonxis, 1949; White, Delory, and Israels, 1950; Betke, 1951; Kleinknecht, 1953). Betke (1952a, 1954) has shown neatly and conclusively that normal adult haemoglobin is not divisible into fractions of varying lability to alkali by dialysing the filtrate in a precipitation-denaturation experiment and showing it to be identical in its behaviour towards alkali with the original sample brought to the same concentration.

That different results are obtained by the two methods commonly used for following alkali denaturation, viz., the optical and precipitation methods, is well known (e.g. Jonxis and Huisman, 1956) and was indeed to be anticipated. Itano has shown furthermore (1957b) that, if the reaction is followed spectrophotometrically at different wavelengths, different rate curves are obtained. Each of these may correspond, though not uniquely, to concentration changes of the oxyhaemoglobin, alkaline haematin, or any intermediate products. The interpretation of such data in terms of the fate of the native protein is problematical. The precipitation method is equally arbitrary in its choice of a criterion for denaturation, and has been criticized on other grounds (Itano, 1957b), in particular that the precipitate might carry down with it some undenatured haemoglobin. This has not been the experience of Betke (1953a), who has shown that no haemoglobin is released from the precipitate by dilute ammonia. Betke also gives an interesting comparison between the reaction rates of oxy- and carboxyhaemoglobin as indicated by the two methods. Penati, Turco, and Lovisetto (1954c) give an equation relating the results obtained by the two methods.

Kubowitz (1954) has suggested that loss of oxygen or carbon monoxide affinity is a more satisfactory criterion of denaturation, and it does indeed seem particularly suitable for the study of haemo-proteins (v. also Kubo, 1956). Moreover, as a means of following alkali denaturation reactions, his manometric method is less ambiguous than the usual procedures. The rate of reaction as observed by this means is greater than that derived from optical measurements at the usual wavelengths. Kubowitz regards the values from the optical method as incorrect. One fundamental point appears, however, to

have been neglected, namely the possibility discussed below that methaemoglobin is formed in the first stage of the denaturation process. This would naturally lead to a loss in oxygen-binding capacity, even though the mere formation of methaemoglobin could scarcely be said to be consistent with any of the accepted definitions of denaturation. Kubowitz has in addition carried out measurements on reduced haemoglobin where the rate of formation of the end product (haemochromogen), as followed spectrophotometrically, agrees with the gasometric measurements, and has studied crystallized Hb-F and Hb-A, which were found to behave in the same way as the corresponding red cell haemolysates. Kubowitz has also been at some pains to show that the quantitative determination of Hb-F in admixture with Hb-A is to be relied on only when the proportion of Hb-F is substantial. This is borne out by the observations of Beaven *et al.* (1956), and observations of the presence of small alkali-resistant components in normal adult blood (Singer *et al.*, 1951; Huisman, Jonxis, and Dozy, 1955; Künzer, 1955) must be judged accordingly. It is interesting to note in passing that the isolated globin of Hb-F is abnormally prone to denaturation (Havenga, 1953).

Besides Hb-F two other characterizations of haemoglobins in terms of alkali denaturation rates have been claimed. The first is the pre-foetal haemoglobin (Hb-P) of Allison (1955). A haemoglobin was found by Halbrecht and Klibanski (1956) and Halbrecht, Klibanski, Brzoza, and Lahav (1958) in young fetuses which they claimed to be highly alkali-resistant. On the other hand, Künzer (1957b), Künzer and Drescher (1956), and Drescher and Künzer (1953, 1954) describe their pre-foetal component found under the same circumstances to be intermediate between Hb-F and Hb-A in its resistance to alkali. Recently a new "fast" haemoglobin, described as "Bart's" was reported by Ager and Lehmann (1958) in the presence of Hb-F in an infant blood and was stated to show resistance to alkali greater than Hb-A in the Singer one-minute test.

Acid Denaturation.—Attempts have been made to characterize Hb-F by acid denaturation but have produced little more than the fact that the resistance of Hb-F to acids is greater than that of Hb-A.

The rate of change of oxyhaemoglobin-A into acid haematin has been studied by Bugyi (1956), and Kleihauer (1957) has demonstrated by a precipitation method that Hb-F is two and a half to three times more resistant to hydrochloric acid than Hb-A. Putignano and Cognetti (1952) claim that carboxyhaemoglobin from the blood of thalassaemia patients is much more resistant to acids than the normal adult pigment. A more exhaustive study by Penati, Lovisetto, and Turco (1955c) has shown that the precipitation method cannot profitably be applied to acid denaturation since the differences in the rate curves for Hb-F and Hb-A are too small to give

reliable extrapolated concentrations. Differences between the acid denaturation curves can be obtained by following the reaction spectrophotometrically, but are regarded as insufficient to permit of any useful conclusions.

Heat Denaturation.—This procedure has been applied by Betke and by Betke and Greinacher (1954) to the characterization of Hb-F (Betke, 1953c; Betke and Greinacher, 1954b) and Hb-S (Betke and Greinacher, 1955). The reaction is first order and indicative of homogeneity in Hb-A, Hb-F, and Hb-S. Mixtures of haemoglobin F and A give a denaturation curve characteristic of heterogeneity which can be expressed as the sum of the individual reactions. Hb-F is more susceptible to heat denaturation than Hb-A, differing by a factor of two to two and a half at 72° C. Similar behaviour is exhibited by the cyanmet derivatives. Hb-S shows no difference in its behaviour from Hb-A.

Other Denaturing Agencies.—The denaturation of haemoglobin with urea and with sodium salicylate has been studied in a number of connexions (*v. infra*).

A comparative study of methaemoglobins F and A was made by Gardikas, Scott, and Wilkinson (1953), who found that Hb-F was more resistant to sodium salicylate, but less so to urea.

Oxygen Dissociation.—The oxygen dissociation curves of the haemoglobins have been the subject of intensive study in virtue of their physiological interest.

Since the oxygen-binding capacity of haemoglobin is essentially a function of the haem iron, it might be considered surprising to find differences in affinity arising from aberrations in the globin portion. On the other hand it is now known (Riggs, 1952) that the haem-haem interactions which appear largely to govern the shape of the oxygen dissociation curve (see for example Wyman, 1948; St. George and Pauling, 1951) are strongly dependent on the sulphhydryl groups in the molecule, and these in turn will be affected by any fairly drastic treatment of the pigment. It should be possible to explain in these terms the early observations of Geiger (1931), who separated animal and human haemoglobins cataphoretically into two components, present in bulk, which exhibited markedly different oxygen equilibrium curves. Moreover, the presence of unknown erythrocyte constituents (Allen, Wyman, and Smith, 1953; Becklake, Griffiths, McGregor, Goldman, and Schreve, 1955), as well as pH and ionic strength (Takashima, 1955, etc.), have their effects on the oxygen dissociation equilibrium, and consequently specific differences between, for instance, haemoglobins F and A in this respect (e.g., McCarthy, 1943) cannot at the present state of knowledge be accepted without reserve. Indeed, Allen *et al.* (1953) have shown that whereas fresh solutions of Hb-F gave values of oxygen

affinity consistently less by about 13% at the half-saturation point than Hb-A after simultaneous dialysis of the haemolysates from foetal and maternal blood against a common volume of buffer the oxygen affinity of the two haemoglobins became equal, the Hill constant remaining unchanged.

Oxidation and Reduction Reactions.—Künzer and Künzer (1952) observed that spontaneous oxidation to methaemoglobin occurred in cord blood more rapidly than in adult or infant blood. This has also been observed by Betke, Kleihauer, and Lipps (1956d).

Detailed kinetic studies of the oxidation of adult, foetal, and other haemoglobins have been carried out by Betke and, in the case of oxidation by molecular oxygen, a reaction evidently of some complexity, by Kiese and Schneider (1955). It was noted by Betke (1952b, 1953b) and by Künzer and co-workers (Künzer, Ambs, and Schneider, 1953; Künzer and Saffer, 1954) that foetal haemoglobin was oxidized more rapidly to methaemoglobin than the adult pigment. Künzer and Saffer give the ratio of half-reaction times with potassium chlorate as 1.59:1, and large differences with sodium nitrite are reported (Künzer *et al.*, 1953). Betke (1952b) and Betke, Greinacher, and Hecker (1956) followed the oxidation of reduced adult and foetal haemoglobins by potassium ferricyanide under various conditions of pH and at different initial concentrations of haemoglobin and methaemoglobin by an optical method, and found that under their conditions there were no perceptible differences between the behaviour of the two haemoglobins. Betke (1953b) also followed the reduction of the methaemoglobins by sodium dithionite by a streaming method, and showed that within the accuracy of his apparatus, which he gives as 5%, the behaviour of Hb-A and Hb-F was identical. The reduction of the oxyhaemoglobins was observed in the same apparatus, and, if the first-order mechanism originally proposed by Hartridge and Roughton (1925) is assumed, the reaction half-times of adult and foetal oxyhaemoglobins under these conditions are 0.015 and 0.021 seconds respectively.* The implication is clear: the molecules are protected by bound oxygen against oxidation and, under the given conditions, the oxygen binding of adult haemoglobin is more firm than that of foetal. This is confirmed by the fact that oxidation of foetal oxyhaemoglobin with sodium nitrite proceeds at twice the rate as that of the adult pigment, though in their reduced forms the haemoglobins again show no difference. Betke and Scholz (1958) have shown that the effect does not arise from differences in saturation of different haemoglobins at a given pressure.

A full account of Betke's work on the oxidation of animal haemoglobins, as well as the human

haemoglobins A, F, C, and S (the last of which was investigated also by Betke and Greinacher (1955)), is to be found in an interesting recent paper (Betke, 1957). The reaction with ferricyanide appears to follow first-order kinetics, and, of the haemoglobins examined, heterogeneity is evinced only by human cord blood haemoglobin and by guinea-pig haemoglobin (Betke *et al.*, 1956a; Betke, 1957). The postulate of a summation curve due to two components is shown to give results for cord blood haemoglobin which are consistent with the results of alkali denaturation analysis. No differences are observed between haemoglobins A, C, and S. This is scarcely surprising since no differences in their oxygen-binding capacities have been discovered (Wyman and Allen, 1951). Betke finds that the oxidation of haemoglobins by nitrite ion is more complex, and advances evidence in favour of an autocatalytic mechanism (Betke, Greinacher, and Tietze, 1956b; Betke, 1957). On the other hand, Remmer and Meyer-Wilmes (1958) find that the reaction with ferricyanide is not stoichiometric, 2.3–2.9 equivalents being required per quarter-mol of haemoglobin. It is believed that another reaction also occurs, in which sulphhydryl groups are oxidized.

Itano and Robinson (1956, 1957, 1958) have reported the oxidation of carboxyhaemoglobin to take place in successive steps, in each of which one haem iron atom is oxidized. Components have been resolved electrophoretically corresponding to these intermediates, and agreeing in their proportions with the results of spectrophotometric analysis.

Itano (N.A.S.-N.R.C. Conference, 1958, p. 151) has suggested that an absolute standard for the correlation of overall charge difference with relative migration rate for different haemoglobins might be available through comparison with such intermediates, which are known *a priori* to differ from each other by one charge unit. Reasoning of this kind indicates, for instance, that the charge difference between Hb-C and Hb-S is smaller than that between Hb-S and Hb-A.

Adsorption

The adsorption isotherms of various haemoglobins would have considerable interest, apart from their bearing on chromatographic behaviour. Only brief studies have been carried out, however, again by Betke and Greinacher, of the adsorption of Hb-A, Hb-F (1954a), and Hb-S (1955) on alumina, partly in connexion with the elimination of stromal materials from red cell haemolysates. Some differences were observed, notably that alumina will take up about 1.5 times as much foetal as adult haemoglobin.

Viscosity

Differences between the viscosities of solutions of Hb-S and Hb-A have been discussed by Allison (1957). The results of such work may be

*Complications arising from the use of sodium dithionite for the reduction of haemoglobin are described by Dalziel and O'Brien (1957). Roughton (in N.A.S.-N.R.C. Conference, 1958, p. 31) states that haemoglobin must not be exposed to dithionite for longer than a fifth of a second if it is not to undergo permanent change.

found to have a bearing on the structural differences between haemoglobin variants, in particular in terms of the disposition of groups responsible for intermolecular association and the role of the sulphhydryl groups. No viscosity differences between the corresponding oxyhaemoglobins are found (Nakamura, 1955).

Sedimentation and Diffusion

A substantial difference between the sedimentation rates of foetal and adult haemoglobins in ultracentrifuge experiments was reported by Andersch, Wilson, and Menten (1944), who suggested on the basis of their results that Hb-F had half the molecular weight of Hb-A. Apart from the lack of support for such a hypothesis from any other experimental source, the sedimentation studies are not in agreement with the diffusion experiments of Taylor and Swarm (1949), who find that no difference exists between haemoglobins A and F. The ultracentrifuge technique has also been used to study dissociation equilibria in haemoglobin, and the results will be mentioned under another heading.

Crystal Structure

The characterization of haemoglobins in terms of their crystal structures has been carried out in recent years by Perutz and co-workers.

The crystallization of adult and foetal haemoglobins was first subjected to detailed study by Jope and O'Brien (1949) and later by Perutz *et al.* (1951), who also crystallized oxyhaemoglobin S. Under suitable circumstances these haemoglobins can give rise to tetragonal or orthorhombic systems. Single crystals of foetal carboxyhaemoglobin of known salt and water content have also been studied by x-ray methods by Zinsser and Tang (1951). Crystallographic interest has naturally centred on the reduced form of Hb-S, which exhibits notably different physical properties from other human haemoglobins (Perutz and Mitchison, 1950). (These will be discussed later under Hb-S.) Crystals of other haemoglobins have also been isolated, e.g., Hb-G and known crystals of Hb-A and Hb-G by Edington, Lehmann, and Schneider (1955), but no detailed analyses of structure have yet been published. Rossi-Fanelli, Antonini, and Mondovi (1957) comment on an anomalous crystal form associated with an abnormal methaemoglobin (apparently from Hb-M).

Absorption Spectra

The absorption spectra of all known haemoglobins have been examined with greater or lesser thoroughness.

The outstanding example of spectral deviation occurs in Hb-F, in which the tryptophan band is displaced toward a shorter wavelength (289.8 m μ) compared with Hb-A (291.0 m μ) and appears as a distinct absorption maximum rather than merely an inflexion (Jope, 1949; Beaven, Hoch, and Holiday, 1951). The analytical value of this observation is discussed in detail by Rich (1952) and by Beaven and Holiday (1952). The remainder of the visible and ultra-violet spectrum of foetal haemoglobin and its derivatives has been examined with great care (Lambrechts and Martin, 1951; Polosa, Motta, and Lo Turco, 1957), and it can be said with some certainty that no other significant differences in the positions of bands exist. Lambrechts and Martin (1951) examined the "Hufner quotients" for a number of wavelengths in the visible region of the Hb-F and Hb-A spectra, but found that intensity differences, if they existed, were less than 3%. Penati, Turco, Grandis, and Lovisetto (1955d), however, have reported that absolute intensity differences, although small, do exist in the ultraviolet, and present absorption curves of mixtures of adult and foetal haemoglobins in various proportions, which support their claims. The differences observed between pure adult and cord blood haemoglobins (ca 80% Hb-F) are of the order of 10 to 20% in the region of the tryptophan and tyrosine bands, with a maximum difference at 289 m μ . Some interesting curves are also given relating extinction coefficients at a number of wavelengths in the tryptophan band region with the percentage of Hb-F in a foetal-adult mixture.

Spectral deviations have been claimed in three other haemoglobins. Künzer and Drescher (1956) state that their pre-foetal haemoglobin, of intermediate alkali resistance, shows a tryptophan band at 289.8 m μ , and a sample supplied by Dr. Lehmann, and said by him to contain a new fast haemoglobin ("Bart's"), again, it may be noted, isolated from a mixture containing Hb-F, was examined with the logarithmic cam spectrograph and found to have a tryptophan band in the position characteristic of an Hb-A/Hb-F mixture in which the latter preponderates (Ager and Lehmann, 1958).

The Hb-M variant comes into a category of its own. This has been observed only as the methaemoglobin, which differs from normal methaemoglobin in features in its visible spectrum, in particular the presence of an absorption minimum, rather than a maximum, at about 630 m μ (Hörlein and Weber, 1948, 1951; Kiese, Kurz, and Schneider, 1956; Heck and Wolf, 1958; Rossi-Fanelli *et al.*, 1957; Gerald, Cook, and Diamond, 1957; Gerald, 1958). This observation, if accepted at its face value, is very extraordinary because it would seem to imply the existence of an aberration in the haem portion of the molecule. Hörlein and Weber (1948), as well as Kiese and Schneider (1955), claim to have shown, however, by splitting the haem-globin linkages and recombining the globin with haem derived from normal adult haemoglobin, that the abnormality is

in fact inherent in the globin. The significance of these observations will be discussed below.

Recently McCord and Gadsden (1958) reported small anomalies in extinction values in the visible spectrum of Hb-S. These anomalies, which were obtained from difference spectra of Hb-S and Hb-A derivatives, occurred, however, only at absorption maxima, at which the actual absorption values were greater by a factor of about 10 than that at the wavelength at which the solutions were matched. Under these conditions, errors in matching are greatly magnified.

Infra-red spectroscopy does not readily lend itself to the study of proteins: a survey of its application and shortcomings in this field is given by Sutherland (1952). A detailed examination of Hb-A in this region is described by Larson and McLaughlin (1955). As is to be anticipated, the comparison of haemoglobin spectra in the infra-red has not met with any great success. Studies on adult and foetal haemoglobins have been carried out by Greinacher, Greinacher, and Betke (1953) in the region between 2 and 15 μ , and on whole blood and haemolysates in various pathological conditions, including thalassaemia major, by Wegmann, Barthel, and Thewes (1957). In neither case were significant differences observed. Penati, Borello, Turco, and Lovisetto (1955b) have carried out similar investigations in the 2–11 μ region and have reported small but consistent differences between the ratios of peak intensities of the polypeptide bands at 7.7 and 8.03 μ in adult and foetal haemoglobins. Further investigation along these lines is unlikely to be profitable.

Differences Arising in Whole Blood

In the main, the behaviour of haemoglobins in whole blood is so heavily influenced by extraneous factors that it lies beyond the scope of this review. A few observations having preparative potentialities or implications shedding light on the physical or chemical specificity of a given haemoglobin species will nevertheless be briefly mentioned.

Centrifugation. — Bromberg, Rabinovich, Abrahamov, and Salzberger (1956) have recorded the interesting observation that the centrifugation of red cells of cord blood gives rise to a layering in which foetal haemoglobin occurs in higher concentration at the bottom than at the top. It may be possible to explain this phenomenon in terms of the results of Simon and Topper (1957) on the centrifugation of cells containing labelled haemoglobin. These workers found that a stratification occurred with the oldest cells at the bottom. Since at birth the rate of erythropoiesis of Hb-A is increasing whereas that of Hb-F is decreasing, so that the average age of cells containing predominantly the latter would be expected to be the greater, the layering in cord blood might be a manifestation solely of cell age and not of haemoglobin type.

Haemolysis Effects.—Although in general the haemolytic behaviour of a red cell is in no sense a function of the haemoglobin type that it contains, the occurrence of haemoglobin variants is frequently accompanied by altered fragility characteristics.

A large number of osmotic fragility studies have been made, and it seems likely that, on the basis of some of the results of such work, a fractionation of haemoglobins from mixtures occurring in blood samples could be achieved. Recent examinations of osmotic resistance of erythrocytes containing foetal haemoglobin have been made by Dettori (1957), by Astaldi, Tolentino, and Sacchetti (1955), and by Sjölin (1954). Erythrocytes containing Hb-S have been the subject of particularly thorough investigations in relation also to their increased mechanical fragility (Lange, Minnich, and Moore, 1951; Griggs and Harris, 1956). Osmotic fragility measurements have also been made on cells containing haemoglobins C (Spaet, Alway, and Ward, 1953), D (Chernoff, 1958), E (Chernoff, Minnich, and Chongchareonsuk, 1954), and H (Spaet *et al.*, 1953; Vella, 1957; Brain and Vella, 1958). The effect of cell age must again be borne in mind (Simon and Topper, 1957).

Immunological Specificity

The specificity of haemoglobins F and A in the precipitin reaction was demonstrated by Darrow, Nowakovsky, and Austin in 1940.

Subsequently the antigenic behaviour of these haemoglobins was examined by Vecchio and Barbagallo (1950), by Goodman and Campbell (1953), and by Chernoff (1953b), who claims by an immunological method to have shown that foetal haemoglobin is present to the extent of 0.05 to 0.5% in normal adult blood (Chernoff, 1953a), by Aksoy (1955), and by Sansone and Durando (1951). Recently Diacono and Castay (1957) have demonstrated specificity of Hb-F by a complement deviation method and have also examined the antigenic behaviour of haemoglobins S and C (1956a, 1956b). Other immunological work has also been carried out on Hb-S, but its characterization by this means is reported to be difficult (Goodman and Campbell, 1953). Summaries of the "double diffusion" technique in agar as applied to human haemoglobins, together with the results obtained, are given by Ruggieri and Marchi (1955) and by Chernoff (in N.A.S.-N.R.C. Conference, 1958, p. 179). An attempt has also been made to analyse human haemoglobins by immunoelectrophoresis (Boivin and Hartmann, 1958).

Genetic Criteria

One case can be cited in which the evidence for the existence of a haemoglobin variant rests very heavily on the genetic circumstances associated with its occurrence. This is the "fast" haemoglobin of Fessas and Papaspyrou (1957), which

was found in a child whose parents were suffering from thalassaemia and thalassaemia trait. Its behaviour in paper electrophoresis was only subtly different from that of haemoglobins I and J, but distinct from Hb-H. The genetic manifestations are consistent only with the behaviour of Hb-H (Motulsky, 1956). This being ruled out, the authors conclude that the new entity is distinct from haemoglobins I and J, and is a hitherto unknown form.

Structural Differences

Amino-acid Composition.—It is not proposed to analyse the results of the numerous terminal group and total amino-acid analyses which have been made on the human haemoglobins.

It must be noted that some of the discrepancies between the results of different workers seem to arise in vagaries in Sanger's dinitrophenyl technique. An examination, for instance, of the end-groups of Hb-A by Brown (1952) indicated in the first place two valine and 43 lysine ϵ -terminal groups, but with other, seemingly more reactive preparations of the dinitro-fluorobenzene reagent further reaction occurred, giving in all some five valine residues. Rhinesmith, Schroeder, and Pauling (1957b) find that Hb-A has four N-terminal groups, all valine. Terminal groups have been examined by Masri and Singer (1955), applying the Sanger DNP method to haemoglobins A, C, F, and S, by Havinga (1953) working with haemoglobins A and S, by Schapira and Dreyfus (1954) on haemoglobins derived from subjects suffering from thalassaemia, by Huisman and Drinkwaard (1955) on haemoglobins A, C, F, E, and S, and by Huisman and Dozy (1956) on haemoglobins A, C, F, and S by digestion with carboxypeptidase.* The amino-acid nitrogen contents of haemoglobins S and A were shown by Schroeder, Kay, and Wells (1950) and by Dickman and Moncrief (1951) to be very similar.

Complete amino-acid analyses on haemoglobins F and A were carried out by van der Schaaf and Huisman (1955a), who obtained the curious result that the alkali-resistant fraction in the haemoglobin of sickle-cell anaemia is not the same as Hb-F, by Dustin, Schapira, Dreyfus, and Hestermans-Medard (1954), using globin derived from Hb-F, by Rossi-Fanelli, Cavallini, and de Marco (1955), by Huisman, Jonxis, and van der Schaef (1955) on haemoglobins A, S, F, and C, and by Jonxis, Huisman, van der Schaef, and Prins (1956) on Hb-E. The most interesting result which has accrued is the discovery of a large difference in the content of *isoleucine*, which is present in quantity only in Hb-F (van der Linden, 1950; Rossi-Fanelli *et al.*, 1954). Derrien and

Laurent (1955a), for instance, find that Hb-F from cord blood has an *isoleucine* content of 1.94%, and that normal adult carboxyhaemoglobin contains 0.34%.

They find further that the so-called alkali-resistant fraction of normal adult oxyhaemoglobin contains 3.25% and the corresponding fraction of adult carboxyhaemoglobin 1.9% *isoleucine*. The presence of two types of alkali-resistant haemoglobin in adult carboxyhaemoglobin is inferred. The evidence, however, appears a little slender for so remarkable a conclusion. Other minor differences in composition are reported by Rossi-Fanelli, Cavallini, de Marco, and Trasarti (1955c). Recently a most interesting paper has appeared by the Rockefeller group of workers (Stein *et al.*, 1957), in which determinations of amino-acid compositions of haemoglobins A, F, S, and C are described, which are without doubt the last word to date in accuracy and reliability. The haemoglobins were isolated pure and free from minor components by electrophoresis on starch. *Isoleucine* was present only in the minutest traces except in Hb-F. The highest content in the other haemoglobins was 0.45 of an *isoleucine* residue per molecule of Hb-C. It is clear, therefore, that the *isoleucine* content of the haemoglobins examined, other than Hb-F, arises from impurities. Moreover, a simple calculation shows that if this impurity, when detected in normal adult haemoglobin, were Hb-F, the *isoleucine* content is such as to indicate its presence in quantities from 10 to 25%. It is to be concluded categorically, therefore, that the impurity in question is not Hb-F. It is conceivable that the *isoleucine* derives from the colourless component described by Derrien (1957; Derrien, Laurent, and Borgomano, 1956) and associated with the so-called alkali-resistant fraction of normal adult haemoglobin, said by him to contain some 4% *isoleucine*. Rhinesmith *et al.* (1957a) reported that heterogeneity in normal adult haemoglobin disappeared on crystallization, but Allen *et al.* (1958) found that heterogeneity on ion exchange columns remained, though the *isoleucine* content of the protein was unchanged by crystallization (but cf. Rossi-Fanelli *et al.*, 1955b), though different portions of the column eluates contained widely different amounts of *isoleucine*, and some indeed none at all. The drastic effect which impurities or minor haemoglobin components can have on amino-acid composition is thus evident.

Schroeder and Matsuda (1958) have also found that Hb-F and Hb-A contain equal numbers of glycol residues, as well as valyl N-terminal groups. It is suggested that the N-terminal sequence valyl-leucyl occurs in both Hb-A and Hb-F, and that these proteins may each have one identical chain containing such a sequence, though differing in the other chain which terminates in glycine in Hb-A and a valyl-histidyl-leucyl sequence in Hb-F.

A significant development in the molecular analysis of haemoglobins has been developed by Ingram (1957; Hunt and Ingram, 1958b), who has devised

*The results of these workers for Hb-A are not altogether in accord with the C-terminal amino-acid determination of Kauffmann and Boettcher (1958), who find that, using hydrazine, two C-terminal histidine groups are released from Hb-A as against one if carboxypeptidase is used. An interesting discrepancy between determinations in haemoglobin and on the isolated globin was noted and is ascribed to the release of free amino-acid in the presence of haem.

means of breaking up the haemoglobin into short-chain peptides, which are examined and identified by two-dimensional paper electrophoresis and chromatography. The composition of these peptides gives a first indication of the order in which amino-acid residues occur in the protein, and the interesting result has emerged that haemoglobins S and C differ from Hb-A in the identity of only one residue.

Hunt and Ingram (1958a) have gone a stage further in explaining the molecular nature of haemoglobins, and have digested the trypsin-resistant "core" of haemoglobins A and S with chymotrypsin. The "core" comprises a considerable portion of the protein, and is poor in lysine and arginine, but rich in aromatic amino-acids, and those with long non-polar side chains. No differences between Hb-A and Hb-S were found.

The method of "differential titration" on filter paper of Scheinberg *et al.* (1954), already alluded to, is interpreted by these authors in terms of free carboxyl groups, but the inconsistency of their results—most notably in the case of Hb-C—with the results of end-group analyses, leaves the validity of their reasoning open to doubt. It has recently been reported (Benzer, Ingram, and Lehmann, 1958) that analysis of three different Hb-D samples by tryptic digestion has shown them to have different compositions. It has been previously pointed out (Vella, Wells, Ager, and Lehmann, 1958) that where a given variant has been obtained from a number of ethnically distinct sources, the identity may be only apparent, resulting from a similarity in electrophoretic and/or chromatographic behaviour. The conclusion then presents itself that if the methods used are reliable there are indeed a great many haemoglobin variants. The number of possible arrangements of units in the protein chains which could give rise to functional haemoglobins might be expected to be very large indeed.

Sulphydryl Groups.—The relation between the free sulphydryl groups in the haemoglobin molecule and the nature of the oxygen dissociation curve (Riggs, 1952) has been mentioned.

Ingbar and Kass (1951) carried out amperometric titrations of haemoglobins A and S and found the former to contain two and the latter three accessible sulphydryl groups. More recently, Ingram (1955) has examined Hb-A and has found by titration with silver nitrate in ammonia that four sulphydryl groups are present. Partial denaturation leads to an increase in the number of titratable groups in Hb-A to eight (Benesch, Lardy, and Benesch, 1955; Ingram, 1955). These results are not confirmed by Allison and Cecil (1958), who describe amperometric titration with mercuric chloride and phenylmercuric chloride, and by making certain assumptions about the nature of the binding of these reagents with the protein arrive at the conclusion that native Hb-A contains two, and denatured Hb-A six, reactive sulphydryl groups. The increase in the number of titratable groups can arise

from the mildest treatment, such as the elevation of the temperature to 38° C., as shown by Murayama (1957a, c, 1958), except in Hb-F (Murayama, 1957b; Hommes, Santema-Drinkwaard, and Huisman, 1956). It is clear that this behaviour is governed predominantly by steric factors. The configurational implications are discussed by Murayama (1957a). (For criticisms of his deductions, see N.A.S.-N.R.C. Conference, 1958, pp. 238–252.)

Hommes, Dozy, and Huisman (1958) have examined Hb-F and find that, of the six half-cystine residues present, four are in the form of reactive sulphydryl groups. After reduction with thioglycolic acid, the protein is found by electrophoresis to have been split into two components. The presence of two different polypeptide chains joined by one or more disulphide bridges is inferred.

Stein *et al.* (1957), working with their electrophoretically purified haemoglobin samples, find four to five sulphydryl groups per molecule of haemoglobin, except in Hb-F, for which they find a value of 3.4, and they state also that determinations of the half-cystine content of the haemoglobin examined agreed with the amperometric titrations. These authors have observed in addition that treatment with sodium sulphite does not give rise to an increased number of titratable sulphydryl groups. The results of titrations of haemoglobins which have not been purified by electrophoresis, that is to say, in the condition in which they have presumably been examined by other workers (Hommes *et al.*, 1958, etc.), exhibit variations from sample to sample, which are largely removed by electrophoresis. It is concluded that there are present impurities, rich in sulphydryl groups, which are largely separated by electrophoresis on starch.

The condition of the sample is also of importance. It is known that sulphydryl groups are easily oxidized not only by the ferricyanide ion and other reagents (Remmer and Meyer-Wilmes, 1958) but also by molecular oxygen. This has been shown by Tsen and Tappel (1958). Taylor (1955) has shown that ferricyanide oxidation with consequent transformation of sulphydryl groups gives rise to an effectively new molecular species with different solubility and electrophoretic mobility. The results of most of the older determinations of active sulphydryl groups perhaps demand re-examination in this light. Kajita (1956) used an interesting method for the evaluation of free sulphydryl groups, in which oxyhaemoglobin was oxidized with potassium ferricyanide. Aliquots were taken during the reaction and analysed for total consumption of reagent and methaemoglobin formed. By subtraction this gave a value of only one free sulphydryl group per molecule of native Hb-A at a pH of 5.5 to 8.5. This was increased to 2 to 2.5 when the pH was raised to 9.5, and the addition of protein "perturbators," such as benzoic acid in high concentration, gave a further increase to three groups.

It should be noted also that results obtained by different techniques for the estimation of reactive

sulphydryl groups are often not consistent (Hommes and Huisman, 1958). An evaluation of the methods available is evidently necessary.

FACTORS INFLUENCING THE BEHAVIOUR OF HAEMOGLOBIN

A number of properties of haemoglobin will be discussed which, it is thought, may have an important influence on the results derived from the techniques already outlined, and which have so far received little attention in the literature.

Dissociation of Haemoglobin

The first of these properties is the tendency of haemoglobin to dissociate under certain conditions into sub-molecules. This has been carefully investigated by Field and O'Brien (1955) and its effect on boundary spreading in the course of electrophoretic migration is discussed by Field and Ogston (1955). Field and O'Brien, on the basis of sedimentation and diffusion measurements on carboxyhaemoglobin, conclude that the behaviour is independent of *pH* over a range of about 6 to 11. Between *pH* 3.5 and 6 a rapid reversible dissociation into fragments of about half the molecular weight of the haemoglobin molecule occurs, and at lower *pH*, or on prolonged exposure to *pH* 5, this is succeeded by an irreversible denaturation. The dissociation is promoted by dilution as well as by reduced *pH*, and the presence of extraneous substances such as urea.

In 1953 Derrien and Reynaud examined adult carboxyhaemoglobin by free boundary electrophoresis, first in a phosphate buffer at *pH* 6.5 and ionic strength 0.1, and observed the presence of two components, one of them in small quantity. This agrees as far as it goes with Hoch (1950), who, however, observed further small components, of which more will be said later. In cacodylate under similar conditions, however, Derrien and Reynaud observed no less than six components, one of which was a major one. Now this could be related to the artefacts described by Woods (1958) in relation to bovine serum albumin and already referred to, but Itano (1956) has found that the heterogeneity in this case disappears if the cacodylic acid used in the buffer is first recrystallized from alcohol. The implication is that impurities are present which bring about the denaturation of the haemoglobin, with consequent onset of heterogeneity. It would be difficult, however, to explain in the same terms the observations of Shavit and Breuer (1955), on normal adult

carboxyhaemoglobin in cacodylate buffer at *pH* 6.5, that more and more components successively appear as the ionic strength is lowered from 0.1 to 0.005, that is to say, as the concentration of cacodylic acid, with or without impurities, is decreased. They also find that a rise in temperature from 1.5 to 20° likewise gives an increase in heterogeneity.* It is clear that these phenomena might well have a profound bearing on the interpretation of data from free boundary electrophoresis experiments. It is also interesting to note, *à propos* of Itano's observation, the drastic effect which the presence of a minute proportion of an impurity can have on the behaviour of haemoglobin. It might be mentioned that the electrophoretic identification of intermediate stages in the oxidation of carboxyhaemoglobin to methaemoglobin by Itano and Robinson (1956, 1958) was carried out at *pH* 6.85 and the very low ionic strength of 0.01, albeit in phosphate buffer. A number of examples of the characterization of individual haemoglobins by free boundary electrophoresis will be given later.

The possibility of a dissociation equilibrium involving the recombination of sub-molecules derived from parent molecules of different species is also not to be entirely discounted, though recombination of the related sub-molecules would be expected to occur preferentially. Crosswise recombination is believed to occur by Reiner, Moore, Lang, and Green (1942), who observed two fractions in bovine globin at low *pH*, which gave identical absorption spectra, apart from some differences in extinction values. These globins were separable electrophoretically, and also differed in their acid-binding capacity. Their relative proportions indicated that they did not derive from two haemoglobins, and the authors suggested that they arose from recombination of sub-molecules. Moore and Reiner (1944) subsequently extended these observations to the globins of other animals and man. The relative proportions of the fast and slow components changed with *pH*, though with prolonged electrophoresis heterogeneity occurred at any *pH*. The heterogeneity was confirmed by ultracentrifuge experiments at low *pH*, and the Svedberg constants for the two components thus obtained agreed well with those of the two fractions isolated electrophoretically which were

*It has also been claimed that dissociation of part of the oxyhaemoglobin in solutions at *pH* values near the iso-electric point occurs in the presence merely of sodium chloride (Benhamou and Weill, 1956, 1957), though the method used, that of light scattering, is of doubtful applicability. Indeed, other workers (Putzeys *et al.*, 1955) using the same technique find that, so far from dissociation, aggregation occurs under these conditions.

themselves homogeneous. The disintegration of horse haemoglobin into four subunits of roughly equal mass but different mobilities was also shown by Reichmann and Colvin (1956). On the other hand, Haug and Smith (1957) and Smith *et al.* (1957) have evidence that the four subunits are of two types, differing in amino-acid composition. It may also be mentioned that Lewis (1954) and Hughes (in N.A.S.-N.R.C. Conference, 1958, p. 62) have observed that half the haem groups of haemoglobin are more easily removed than the remainder.

Steinhardt (in N.A.S.-N.R.C. Conference, 1958, p. 49) gives reasons for supposing that human haemoglobin is dissociated further, into four fragments, at pH 2.8 at 0° C.

Haemoglobin Binding

The problem of impurities, which is raised by Itano's observation on the heterogeneity induced by impure buffer salts, must now be pursued.

In 1954 Anderson and Griffiths recorded their observations on haemoglobin samples from subjects carrying the sickle-cell trait. The mobilities found for the components in free boundary electrophoresis in phosphate buffer at pH 7.5 and ionic strength 0.1 were not constant. Over nine samples the mobilities of the Hb-A component varied between -1.2 and -2.4×10^{-5} and that of the Hb-S component between -1.7 and -3.8×10^{-5} cm./sec./v./cm. Similar variations were seen on paper. These results, it was claimed, were not artefacts because the figures from a given sample were repeatable, and it was shown spectrophotometrically that methaemoglobin was in all cases absent. Anderson and Griffiths suggested on the basis of these results that apparent heterogeneity in haemoglobins might in fact be due to binding of the haemoglobin with other blood constituents, in particular, haptoglobins and nucleic acids. Mixtures of haemoglobin with nucleic acids were accordingly made, dialysed against buffer, and run in a Tiselius electrophoresis apparatus. The velocity of the haemoglobin was altered and a new peak observed. Similar results were obtained with carbonic anhydrase and with globin (though in the latter case the result of threefold heterogeneity is incompatible with the successful use of globin in admixture with haemoglobin to prevent adsorption of the pigment in paper electrophoresis as reported by Hoch and Barr in 1956). It was also found that, if samples of sickle-cell trait haemoglobin were dialysed before electrophoresis, two new components of intermediate mobility appeared, which were

ascribed to dissociation and altered recombination of the complexes. Though the inferences of Anderson and Griffiths are perhaps a little extravagant, their work nevertheless deserves notice, since binding with various materials in the haemolysates does occur. This would be expected to be particularly severe where whole blood haemolysates are used, or devices are employed such as the partial separation of serum proteins by lead subacetate. That such a process should be recommended for the preparation of haemoglobin solutions for electrophoresis (e.g., Tompkins, 1955) is indefensible, and indeed any results of experiments in which whole blood haemolysates or extracts from unwashed erythrocytes or homogenized clots (Schneider, 1956) are used are open to criticism. It is known that some serum proteins combine most tenaciously with haemoglobin. Foremost among these are the haptoglobins, which have been described by Jayle *et al.* (1952), Jayle and Boussier (1955), Wieme (1953), Reich (1956), and other authors. Binding with these substances, which vary with the individual (Smithies, 1955), substantially modifies the electrophoretic mobility. Tuttle (1955) found that these substances were absent in the blood of newborn children. It is also known that haem groups can combine with the albumin fraction of human serum (Keilin, 1944; Rosenfeld and Surgenor, 1950; Schwerd, 1958; etc.) to give methaemalbumin, and interaction with globulin fractions has also been described (Schwerd, 1958). Binding of serum proteins can vitiate the results of alkali denaturation experiments (Künzer, 1955), and it is noteworthy that the colourless proteins separated by Derrien (1957; Derrien *et al.*, 1956) from Hb-A were found to be associated with the "alkali-resistant" portion. A spectroscopic study has been made by Betke, Greinacher, and Leber (1954), who make the very feasible suggestion that the so-called alkali-resistant component which has been reported from time to time to be present in normal adult haemoglobin (Singer *et al.*, 1951; Huisman *et al.*, 1955; Künzer, 1955, 1957a, etc.) is in fact a complex between the pigment and serum protein. The severe interference in Hb-F estimations caused by the presence of plasma is described by Rappaport, Eichhorn, Konforti, and Roessler-Wolfson (1957). Iversen and Larsen (1956) found that whole blood haemolysates from patients in various anaemic conditions gave alkali denaturation curves of the type associated with Hb-F. This effect vanished when haemoglobin solutions

prepared from washed erythrocytes, and separated from stromal materials, were used. The effect is evidently due to binding with substances deriving from the stroma or plasma. Binding with stromal material, presumably lipoproteins, was shown to occur by Ponder (1952), who found that the complexity of the paper chromatography pattern of adult haemoglobin increased when stromal material was present. Binding of this kind was also demonstrated by Dervichian, Fournet, Guiner, and Ponder (1952b), who examined the orientation of haemoglobin molecules on stromal fragments. It has also been noted (Baar and Hickmans, 1941) that a spurious alkali-resistant component appears in haemolysates which have been prepared with the aid of saponin. A further potential source of minor alkali-resistant components is the presence of carboxyhaemoglobin from endogenously formed carbon monoxide (Sjostrand, 1958). An interesting source of a similar phenomenon is contact with rubber (stoppers, etc.), which has been described by Raper (1957).

The binding of haemoglobin with nucleoproteins is known to be possible (Nelson, 1958), and such substances are present in leucocytes and reticulocytes (Holloway and Ripley, 1952). Various other phosphorus compounds occur in mature erythrocytes (Rottino, Hoffman, and Albaum, 1952; Kutas and Stützel, 1958; etc.). Moreover, Havinga (1953) has shown that Hb-S, unlike Hb-A, has a small phosphorus content, which may be due to bound nucleic acid. Again, Breuer, de Vries, Peket, and Matoth (1957) find that the onset of heterogeneity effects in free boundary electrophoresis occurs earlier, that is to say, at higher ionic strength and lower temperature, in haemoglobin solutions from subjects in various pathological conditions, such as spherocytosis, not associated with abnormal haemoglobins. These authors suggest that this behaviour may be governed by protein constituents in the erythrocytes which remain in the haemolysate and which may be absent in pathological disorders.* It is not inconceivable that the minor components of normal adult haemoglobin which have been observed by a large number of authors (see below) may come about in this way, especially when the decrease in the proportion of secondary component in solutions on standing, as observed by Berry and

Chanutin (1957, 1958), is borne in mind. Changes in the heterogeneity pattern associated with microcytosis (Modiano, 1957; Silvestroni and Bianco, 1957), etc., and the curious small components of Masri *et al.* (1958) may also be noted. Some indication in this direction is also provided by the remarkable observations of Cabannes, Raffi, and Boineau (1957a) on subjects in a family suffering from a haemolytic disorder. These authors claim to have separated an A₂ component by starch slab electrophoresis which is present in three members of the family to the extent of 21, 27, and 14% respectively. It is alleged to have been differentiated from Hb-E by its electrophoretic behaviour at pH 6.5. The extraordinary observation by Cook and Morrison (1956) that their minor component isolated from cord blood haemoglobin, running on the columns in the same position as A₂, is completely resistant to alkali under conditions where foetal haemoglobin itself is labile provides a further pointer.

The separation of colourless proteins from various haemoglobin solutions will be seen to have a further bearing on this problem and on the subject of electrophoretic mobilities in general. The content of material other than haemoglobin in the erythrocyte has been closely studied. Stern, Reiner, and Silber (1945) give as the main contents of the non-nucleated erythrocyte 87–95% haemoglobin, 5–12% of other proteins, 0.6% lecithin, about 0.4% cholesterol and varying traces of inorganic substances.* These constituents would be expected for the most part to remain in solution when the cells are haemolysed and centrifuged. The presence of serum proteins and possibly bilirubin in the haemolysate even from washed cells is not to be excluded, since the adsorption of such substances on the stroma (in the same way as haemoglobin is known to be adsorbed (Ponder, 1951)), followed by desorption on haemolysis, is possible. Certainly very little serum protein would be needed to give rise to the spurious effect of a perceptible proportion of alkali-resistant haemoglobin in normal adult blood (Künzer, 1955; Iversen and Larsen, 1956). These cell constituents would be either bound or else in any case too large to be removable by dialysis. Their presence in a haemoglobin solution can be detected electrophoretically and sometimes by other means. Muller and Zijlstra (1957), for instance, found that when they attempted to prepare solutions of known methaemoglobin

*The possibility is mentioned that a tendency for the haemoglobin molecules to dissociate particularly readily may be responsible for the haemolytic disintegration of the erythrocytes in some pathological conditions.

*A more detailed account of erythrocyte constituents is given by Behrendt (1957).

contents for analytical purposes by mixing methaemoglobin and oxyhaemoglobin solutions the proportion of the former was reduced, an effect attributable only to the presence of the methaemoglobin reductase enzyme in the solution. A non-haem coloured protein can be separated, though with some difficulty, by electrophoresis (Lonn and Motulsky, 1957; Masri *et al.*, 1958). Lonn and Motulsky (1957; N.A.S.-N.R.C. Conference, 1958, p. 216) believe this to be methaemoglobin reductase. A putative methaemoglobin reductase has also been separated by Morrison (in N.A.S.-N.R.C. Conference, 1958, p. 167) on ion exchange columns. The separation of non-haemoglobin proteins has also been described by Giri and co-workers (Giri and Pillai, 1956; Giri *et al.*, 1958), who used electrophoresis on agar followed by amido-black staining. It is also a matter of common observation that a clear centrifuged haemoglobin solution will often precipitate stromal material on large dilution or pH or temperature change (Ponder, 1942).

The bearing of the presence of dissolved matter of one kind or another on the nature of the solubility curve has already been touched on in discussing the work of Karvonen and Leppänen (1952b), and the lecithin constituent of the erythrocytes suggests itself as a likely protective colloid. The efficacy of substances of this kind as emulsifying agents is well known. It would be surprising if the presence of these various impurities were not in fact reflected in the solubility curves. Another relevant indication is the fact that, whereas sickling in erythrocytes containing Hb-S occurs when the proportion of this pigment is 7%, for gelling in solution 10% is required. This has been shown by Singer and Fisher (1953), who conclude that the solubility is governed partly by intra-erythrocytic substances.

The work of Roche, Derrien *et al.*, already mentioned (Roche and Derrien, 1951, 1953; Roche *et al.*, 1952, 1953, 1954), could well bear re-examination in this light. Moreover, Betke (1957) has found that the course of oxidation of haemoglobin solutions with sodium nitrite depends markedly on the manner in which the solution has been prepared, whether by simple haemolysis of the red cells, by haemolysis followed by adsorption of extraneous material, or by haemolysis and adsorption followed by dialysis. It is also found that oxidation proceeds differently when crystallized haemoglobin is used (Remmer and Meyer-Wilmes, 1958). Another possible interpretation of this phenomenon will be discussed below.

The separation of colourless proteins from haemoglobin in electrophoresis experiments has been achieved on a number of occasions. Stern *et al.* (1945) isolated a colourless, opalescent component from normal adult haemoglobin by free boundary electrophoresis. This constituted some 2-5% relative to the haemoglobin, and was shown by the Tyndall effect to consist of large particles. It may therefore be a macromolecular protein, a lipoprotein, or stromal material in general. Its mobility is stated to be reasonably consistent with the latter explanation. Giri and co-workers (Giri and Pillai, 1956; Giri *et al.*, 1958) report that three non-haem protein constituents are separable by electrophoresis on agar of red cell haemolysates, and Allen *et al.* (1958) find one such substance associated with one of the haemoglobin constituents fractionated on their ion exchange columns. Slow-running, pale-coloured substances have been isolated on starch blocks by Masri *et al.* (1958) (associated with the "A₄" haemoglobin component) and by Kunkel *et al.* (1957). On paper also, peaks are observed by densitometry in the trail of the haemoglobin zones (Goldberg, 1957), and de Traverse *et al.* (1958) find that a slow moving band is clearly visible after amido black staining. Derrien *et al.* (1956) found a colourless protein present to the extent of 1.3 to 1.5% in normal adult haemoglobin solutions. This is said to be associated with the so-called alkali-resistant fraction of the haemoglobin, separable from it by paper electrophoresis and constituting 43% and 24% of the alkali-resistant fraction when separated respectively from the oxy- and carboxy forms. This result is compatible with the possibility, as outlined above, of complex formation with protein materials leading to an apparently alkali-resistant pigment. The behaviour of this system as described by the above-mentioned authors suggests that an equilibrium may be involved. A minor component of unknown nature has also been observed in Tiselius electrophoresis of mixtures containing Hb-S and Hb-C (Sturgeon, Itano, and Valentine, 1952; Singer, Kraus, Singer, Rubinstein, and Goldberg, 1954).

Zinsser (1952) has found that the presence of the caprylate ion not only alters the mobility of Hb-F in a free boundary apparatus substantially but also caused a colourless protein to be displaced from it. This may well be a bound component, since Zinsser has shown that it does not arise from haem displacement. (It should be noted, however, that fatty acid ions can behave

as denaturants.) Another similar observation may also be mentioned here, viz., the appearance of a precipitate when solutions of haemoglobins K and N are diluted with a cacodylate buffer (ionic strength 0.1) (Robinson, Zuelzer, Neel, Livingstone, and Miller, 1956). Goldberg (1957) notes that in paper electrophoresis the amount of material remaining at the point of application is always greater when abnormal haemoglobins are present. The profound effect of extraneous matter in haemoglobin solutions is emphasized very strongly by the results given in the paper already referred to (Stein *et al.*, 1957) by the Rockefeller group of workers, who found that the isoleucine content observed hitherto in Hb-A was in fact due to an impurity which was not Hb-F. Differences between crystallized and uncrystallized haemoglobin have also been noted (Rhinesmith *et al.*, 1957a; Rossi-Fanelli *et al.*, 1955b), and Allen *et al.* (1958) have obtained fractions from their ion exchange columns which differ widely in their isoleucine content. It is possible that substances varying rich in isoleucine are bound to some of the haemoglobin, particularly when the differences (if real) between the isoleucine contents of oxy- and carboxyhaemoglobin A (Derrien and Laurent, 1955a; Allen *et al.*, 1958) are considered, and that these complexes separate on columns. The particularly high content of isoleucine in the trailing edges of chromatographic and electrophoretic zones may be related to the presence of non-haem proteins in these regions in electrophoresis (Giri *et al.*, 1958). Still more impressive is the evidence (Stein *et al.*, 1957) that large variations in the number of titratable sulphydryl groups arise from foreign substances which are rich in sulphydryl.

It is also interesting that the incubation of proteins with amino-acids under conditions similar to physiological ones causes binding, a good deal of which is due to adsorption but some of which is firm, the amino-acid being inseparable by dialysis (Cornwell and Luck, 1958). The amount of such binding is a function of the pH and also of the amino-acid concentration in the system. Since it is known (Iyer, 1957, 1958) that in some pathological conditions the level of free amino-acids in erythrocytes is increased, it would not be surprising if haemoglobin-amino-acid complexes were to be found in haemolysates. These would behave presumably as separate species. It will be seen, therefore, that the degree of purity of haemoglobins is of the first importance in assessing their behaviour

in any respect and that bound or unbound impurities can give, and no doubt have given, rise to a great many specious results. The apparently anomalous haem-protein ratio of the A₂ component, as examined by Kunkel and co-workers (Kunkel *et al.*, 1957; Ceppellini, 1956), might be regarded as support for the binding hypothesis, as indeed might the observation that the elution curves of Allen *et al.* (1958) from ion exchange columns appear to give different proportions for the minor component of Hb-F, when the concentrations are measured respectively at 280 and 415 mμ. Derrien *et al.* (1956) take a similar view of their minor component.

The nature of the inorganic ions in the erythrocyte, and subsequently in the solution, though their concentration would be small, might well have an effect not only on the solubility curve (Karvonen and Leppänen, 1952a), but also on the electrophoretic behaviour, which is known to depend in large degree on the ions which are present (e.g., Shavit and Breuer, 1955; Morrison *et al.*, 1954; Shooter and Skinner, 1955). The effect of various ionic species on the behaviour of the haemoglobin has been all too little studied. Examinations of rather limited scope have been made by Morris and Wright (1954) and by van Os and Möller (1958), and Scheler (1957) has discussed in great detail the influence of various ions on equilibria in methaemoglobin solutions. Amongst other conclusions which emerge is the likelihood that a part of their action is due to colloidal stabilization. In general it may be said that interactions of haemoglobin with inorganic ions or other dialysable constituents is by no means as problematical as binding with proteins and like materials* though the effect on oxygen dissociation has already been dealt with. The nature of the dialysable substances, on which the shape and position of the oxygen binding curve so heavily depend (Geiger, 1931; Allen *et al.*, 1953; Becklake *et al.*, 1955), is obscure, though some speculations have been made by Hořejší and Komárková (1958). Their further investigation would be of some interest, especially in relation to the striking differences pointed out by Riggs and Tyler (1958), in the slopes of the graphs relating log (oxygen tension at half saturation) and log (body weight) for a number of mammals.

*One case at least is known, however, in which binding with an ionic species is of a very firm nature. Remmer (1958) has found that methaemoglobin, prepared by the use of ferricyanide, contains twice as much iron as is indicated by the pigment concentration as measured spectrophotometrically. The excess iron is not removable by dialysis and Remmer gives reasons for supposing that it is in the form of ferrocyanide co-ordinatively bound to the globin.

These are in contrast with the minute species differences between mammalian haemoglobins, as reflected in their absorption spectra, etc.

Interaction between Haemoglobins

Interactions between different haemoglobins have often been observed, and are in many cases of a quite firm nature. Such interactions can result in anomalous migration velocities in free boundary or zone electrophoresis and in incomplete separation.* The work described above on the results of column fractionation of haemoglobins A and F is presumably a similar manifestation.

The failure of haemoglobins A and F to separate on paper and various other media must arise from an interaction between the two proteins. Itano *et al.* (1956) suggest that this interaction may cause the trailing observed on paper, though this is perhaps rendered unlikely by the work of Hoch and Barr (1956) with haemoglobin/globin solutions mentioned above. Instead of separation on paper a single zone only is to be seen, the speed of migration of which depends on the proportion of Hb-F in the mixture, and has in some cases been used to estimate this proportion (Gatto and La Grutta, 1955a). Zinsser's measurements on the migration of haemoglobins derived from foetal blood under free boundary conditions showed that the younger the foetus from which the haemoglobin had been drawn, the faster the rate of migration of the solitary haemoglobin zone at pH 6.98 in phosphate buffers of ionic strengths between 0.08 and 0.15 (Zinsser, 1952). Further reports of modified mobilities of haemoglobins (which might well be attended by only partial separation) have been made: Edington *et al.* (1955) find that the electrophoretic mobility on paper of Hb-G varies with the other haemoglobins present, and Vella *et al.* (1958) report that the migration of Hb-A on paper is elevated in the presence of Hb-J. Bergren *et al.* (1954) have noted the interaction between haemoglobins C and S, whereby Hb-S migrates more slowly in the presence of Hb-C, and Silvestroni and Bianco (1957) find that the migration velocities of the minor components of Hb-A are changed in the presence of Hb-C.

A striking example of mutual interaction is given by Ramirez and Dessauer (1957a, 1957b)

and Dessauer, Fox, and Ramirez (1957), who have subjected turtle haemoglobin to free boundary electrophoresis. This haemoglobin consists of two species, one fast and one slow, having identical spectra; they were fractionated by a continuous electrophoresis method. It was found that in equimolar mixtures of the two carboxyhaemoglobins at pH 7.4 the mobility of the slow component was no less than twice that of the pure slow component under the same conditions (-4.0 as against -2.0×10^{-5} cm./sec./v./cm.) whereas that of the fast complement was decreased (-3.6 as against -3.9×10^{-5} cm./sec./v./cm.). More examples exist, but the ones cited should give sufficient indication that surprising results which are open to misinterpretation can be obtained in electrophoresis of haemoglobin mixtures. Mutual interaction of haemoglobins or interaction with other substances would certainly affect the subtle balance of distinction between many haemoglobins by paper electrophoresis, e.g., the "fast" haemoglobins as described by Cabannes, Sendra, and Buhr (1957b).

Shooter *et al.* (1958) have noted that in the resolution of haemoglobins of closely similar mobilities, whether by zone or free boundary electrophoresis, the apparent amount of the leading component is increased and that of the slower diminished. (They suggest that the non-resolution of the so-called A_3 component in the starch block separations of Kunkel *et al.* (1957; Kunkel and Bearn, 1957) is a consequence.) Interaction resulting in imperfect fractionation is thus indicated. It is suggested that similar effects occur in the fractionation of haemoglobins by ion exchange chromatography (see above). Interactions of a different kind also arise in the measurement of solubility when solid solutions occur. Mixtures of haemoglobins F and A display such effects (Itano *et al.*, 1956). An interesting case is the solubility of Hb-S in its reduced state, which is substantially less if haemoglobins A, C, and F, as well as other proteins such as serum albumin, are present (Singer and Fisher, 1953). Allison (1957) has investigated the aggregation of Hb-S in solutions of the reduced form by the measurement of viscosity, and his results lead him to the view that mixed polymers are readily formed by Hb-S with Hb-C, less readily with Hb-A (that is to say, the required proportion of Hb-S is greater), and not at all with Hb-F. A study of gelling in Hb-S solutions containing also haemoglobins A, F, or C has been made by Singer and Singer (1953), Hb-C being found to have the strongest influence on solubility. It may

*Spurious fractionations due to interactions in protein mixtures in general have been reported (e.g., Granzer, 1958). They are said to be due essentially to hydrogen bonding and can be eliminated by the addition of hydrogen bond-breaking substances. There is, however, considerable risk of irreversible denaturation.

be noted that interaction with solid solution formation would be expected to occur only in closely similar chemical species. Itano (1953a) observes that such behaviour occurs with a mixture of, for instance, horse and donkey haemoglobins, but not in haemoglobins of less closely related species (*cf.* also Bangham and Lehmann, 1958).

Effects from Denaturation or Modification of the Haemoglobin Molecule

Jope (1949) examined the absorption spectra in the visible and Soret regions of oxyhaemoglobins which had been exposed for varying periods to denaturing agents, in particular urea. The interesting results emerged that in the early stages of attack the Soret band was slightly modified, with a shift towards shorter wavelengths, whereas the visible bands remained unchanged. The possibility presents itself that in the denaturation process intermediates are formed, which may be regarded as slightly modified haemoglobins, having presumably the essential properties of a native haemoglobin, but behaving analytically as discrete molecular species. Jope's work was carried out under conditions not suited to accurate intensity measurements in the visible region. The authors have attempted to confirm Jope's results with the aid of more accurate optical methods, using in the first instance alkali rather than urea as the denaturing agent. A shift in the Soret peak was indeed found to occur. The peak absorption for oxyhaemoglobin is at 414 $m\mu$, and that of alkaline haematin brought to about pH 7 is at 411 $m\mu$, whereas a sample of oxyhaemoglobin exposed briefly to the action of alkali and neutralized has a maximum at 408 $m\mu$. It follows that this solution is not a mixture of oxyhaemoglobin and alkaline haematin generated by denaturation of a portion of the native protein. It was also found that no perceptible changes in the position of the visible bands accompanied this modification, though their persistence was somewhat altered.* The question now arises whether the spectral shift is due to the formation of methaemoglobin as the first reaction intermediate, for this would be consistent with the change in persistence in the visible region. This possibility is being investigated.

A study was made by Banaschak and Jung (1956) of the action of urea on methaemoglobin. Its first effect is the formation of a product with a characteristic absorption band at 534 $m\mu$ at

pH 7, shifting in more acid solution to 537 $m\mu$ with the Soret peak at 413 $m\mu$, but substantially diminished in intensity. This product forms a cyanide but not an azide, and may well be regarded as irreversibly denatured, since removal of urea leads to immediate precipitation. On the other hand, reduction with dithionite gives a pigment with a band at 557 $m\mu$, which is nevertheless not to be identified with reduced haemoglobin on account of the low intensity and the position (424 instead of 429 $m\mu$) of the Soret band. Treatment with oxygen or carbon monoxide, however, leads to products whose spectra are very nearly identical with those of oxy- and carboxyhaemoglobins. Moreover, when the oxy-derivative is oxidized it gives a substance having a spectrum closely similar to that of methaemoglobin, and combining readily both with the cyanide and azide ions. This provides a striking example of a modified haemoglobin, and provokes interesting speculation on the factors governing the coupling of the azide ion. These presumably involve the partial unfolding of the protein chain in such a way that one of the six necessary iron co-ordination positions becomes inaccessible. Banaschak and Jung have also made the remarkable observation that on longer exposure to urea the methaemoglobin forms a second product, spectroscopically identical with the first, which, however, on dithionite reduction forms a haemochromogen, having the characteristic spectrum and behaviour of such a substance. Tsushima and Kawai (1956), using sodium dodecyl sulphate as a denaturant, likewise find that methaemoglobin gives rise successively to two products having identical absorption spectra in the visible, and separable electrophoretically. These are said to represent discrete stages in the unfolding of the polypeptide chains, resulting in the exposure of nitrogen-containing groups, capable of co-ordinating with haem iron to give a hemichromogen. It would be expected that in cases of mild denaturation, electrophoretic mobilities would be altered by elimination or exposure of charged groups or by changes in their pK values. This compound will enter into an equilibrium with the cyanide ion, in which either the latter or co-ordinated globin is displaced, according to experimental conditions (Tsushima, 1956; Scheler, Jung, and Hoffmann, 1954).

White and Kerr (1957) have studied spectrophotometrically the denaturation of adult and foetal haemoglobins by nicotinamide, guanidine, sodium benzoate, and sodium salicylate as well as urea. The absorption curves obtained before

*Saha (1956) working with chicken haemoglobin reports differences in absorption band positions between the native oxyhaemoglobin, and the dialysed and undialysed alkali-resistant residues.

and after dithionite reduction are different from those arising from alkali denaturation at pH 9–11 and are ascribed to denaturation intermediates.

It is obviously worth pursuing any indications of the appearance of modified forms of haemoglobin elsewhere, bearing in mind the implications that such manifestations might have when related to the separation and characterization of components in haemoglobin mixtures. The existence of slightly modified forms of haemoglobin, which have all the significant properties associated with the native protein, is further confirmed by the products of recombination of isolated haem and globin. That the reconstitution of an oxygen-carrying pigment from heat-coagulated or acid-denatured haemoglobin is often possible has long been known (Mirsky and Anson, 1930; Holden, 1937), though such substances evince marked differences from haemoglobin in its native state.

Okken (1954) found that his preparation of recombined haemoglobin exhibited heterogeneity in free boundary electrophoresis, the fast and slow components being present in the ratio of 3:1. Other preparations of reconstituted haemoglobin have been carried out. Rossi-Fanelli and Antonini (1958), for instance, found that their samples of reconstituted haemoglobin gave oxygen dissociation curves identical with those of the native protein. Rossi-Fanelli, Antonini, and Caputo (1958) describe a reconstituted haemoglobin, which is said to have the physical, chemical, and physiological characteristics of the native substance, and is derived from an electrophoretically pure globin. On the other hand, Kistler, Buri, and Nitschmann (1953) have prepared a similar pigment from globin, which, though electrophoretically pure, is shown in fact to be heterogeneous by the nature of its solubility curve. The work of Joep, Joep, and O'Brien (1949) on the properties of recombined haemoglobin casts especial light on the existence of denaturation intermediates. These authors find that globin as usually prepared from Hb-A has a tryptophan fine-structure band in the 289.0–290.3 $m\mu$ region compared with 291.0 $m\mu$ in Hb-A. This globin can be fractionated by salting out, when the fraction having the tryptophan band at the longest wavelength is the least prone to precipitation. All globin with a tryptophan band of wavelength shorter than 290.6 $m\mu$ was rejected, and the resulting material, when recombined with haem, gave a haemoglobin having the band at 291.0 $m\mu$. It was stated that the globins having the tryptophan band at a

shorter wavelength were none the less in the native state by all reasonable criteria. Joep *et al.* also list the wavelengths of the Soret peak of the various preparations, and it is seen that all of them, with the possible exception of the carboxy-derivative of the sample prepared as above, have bands at slightly shorter wavelength than Hb-A. The corresponding methaemoglobins all have a Soret peak in the identical position with methaemoglobin A. Araya, Nakanishi, Nakajima, and Kaminaga (1955) have also prepared a reconstituted methaemoglobin directly by the recombination of haemin and globin at pH 7. This has a somewhat changed spectrum in the Soret region, although the protein bands are not significantly altered. It is clear then, considering also the electrophoretic heterogeneity of Okken's samples, that modified haemoglobins exist which are to be considered as undenatured but nevertheless behave as separate species. It is an interesting speculation whether such variants might occur pathologically.

An example of the occurrence of a modified haemoglobin is that described by Cook and Morrison (1956); ion exchange chromatography of cord blood haemoglobin gives, according to these authors, at least three fractions, the fastest (about 70%) being Hb-F, the slowest (about 10%) running in the same position as A_2 of normal adult haemoglobin, and the remainder corresponding to Hb-A. The portion of the cord blood haemoglobin remaining undenatured at pH 12.7, having the spectroscopic features of oxyhaemoglobin and remaining unprecipitated by one-third saturated ammonium sulphate solution, was also chromatographed. The extraordinary result emerged that only the slowest zone had completely resisted denaturation, and that the residual Hb-F was eluted more slowly from the column after treatment. Apart from this unexpected behaviour of the slow component, it seems clear that the Hb-F, though ostensibly still native haemoglobin, has been modified and behaves as a new species. Methaemoglobin is not likely to be involved here, since the spectrum is said to be characteristic of the oxy-pigment. It is also a matter of common experience (Morrison and Cook, 1955b; Boardman and Partridge, 1955, etc.) that methaemoglobin separates on ion exchange columns as a well-defined brown zone, more strongly adsorbed than haemoglobins.

The question arises then whether haemoglobin fractions isolated by chromatography and electrophoresis have been sufficiently scrutinized spectroscopically. The modified Hb-F, for

instance, of Morrison and Cook is to have the spectral characteristics of oxyhaemoglobin, but presumably neither these nor other authors in the field have examined their samples sufficiently carefully to show up small spectral differences of the kind observed by Jope and the present authors. It is proposed to show that such modifications of the haemoglobin molecule, which would not be observed by a cursory spectroscopic examination, could be brought about by far less drastic means than those so far mentioned.

In the first place sufficiently strong adsorption on columns (Kruh, 1952; Boardman and Partridge, 1953; Cook and Morrison, 1956, etc.) or supporting media in zone electrophoresis could result in damage to the protein in the same way as is known to happen in adsorption on alumina (Prins and Huisman, 1956a). This possibility could be resolved by rechromatography of individual zones, as carried out by Boardman and Partridge (1955) and by Morrison and Cook (1955b), though in the latter case the shape of the elution curve of the rechromatographed sample is equivocal. Itano *et al.* (1956) suggest that the poor resolution of haemoglobins on paper at pH values of 7 and below is due to denaturation, presumably by adsorption or conceivably by heat. Modiano (1957), in his interesting paper on the separation and estimation of minor components in Hb-A, suggests that the A₂ fraction observed by him on paper (though not resolved by Kunkel *et al.* (1957; Kunkel and Wallenius, 1955) on starch) may be an adsorption phenomenon of this kind.

More particularly, modification of some of the haemoglobin might well result from the haemolysis procedure applied to the initial preparation of the sample from the erythrocytes. Huisman and Schreuder (1957) have examined and compared a number of methods of haemolysis, and have found that none of them leads to any denaturation of the protein (but see also Baar and Hickman (1941) for the effect of saponin). On the other hand, they give no indication of having examined the spectra of their haemolysates sufficiently closely to show up small differences of the kind mentioned.* It has long been known, for example, that freezing can have a deleterious effect on proteins (Nord, 1936; Oncley, Gurd, and Melin, 1950), which is a fact to be borne in mind where freezing and thawing

are used to effect haemolysis; indeed, Berry and Chanutin (1958) found that haemolysates thus prepared gave rise to abnormal patterns in free boundary electrophoresis, a large number of components being obtained. It may be mentioned also that Hb-H is said to be destroyed completely on freezing (Rigas, Koler, and Osgood, 1956). Huisman and Schreuder conclude that the best method of haemolysis is to add toluene and this appears in any case to be the most widely applied technique. Now Betke *et al.* (1956c) have shown that even a brief homogenization of toluene with oxyhaemoglobin solutions produces extensive denaturation, with formation of methaemoglobin in the undenatured portion. It would not therefore be surprising if intermediate species or denaturation products were to be formed, which could give rise to severe artefacts in fractionation experiments. Berry and Chanutin (1958) have in fact reported unsatisfactory results with haemolysates prepared by Drabkin's (1946) procedure. Prolonged exposure to air (Tsen and Tappel, 1958), as well as adsorption on vessel walls and mechanical agitation (Wu and Ling, 1927) or frothing, during, for instance, carboxylation (Nakanishi, Nakajima, Kaminaga, and Araya, 1955), could also be responsible. Before proceeding to examples of observations which suggest an interpretation in these terms, it should also be mentioned that effects of heterogeneity can apparently arise from intermediate oxidation stages between haemoglobin and methaemoglobin. Itano and Robinson (1956) observed the appearance in free boundary electrophoresis of components in partially oxidized carboxyhaemoglobin, which they believe to represent intermediate stages in the oxidation to methaemoglobin, the haem groups being successively affected. Itano and Robinson have more recently (1958) reported the appearance of intermediates in the conversion of methaemoglobin into its cyanide derivative. (This work as it stands is rendered ambiguous, however, by the observed fact (Remmer and Meyer-Wilmes, 1958) that the reaction of oxyhaemoglobin with ferricyanide is far from stoichiometric: according to the results of Remmer and Meyer-Wilmes, the proportions of reagents used by Itano and Robinson should not in fact give the ratios of components that they obtain electrophoretically. It is said (Itano and Robinson, 1958) that if oxidation is carried out at pH values below about 7, no sulphhydryl groups are oxidized. This, however, does not guarantee a stoichiometric reaction with ferricyanide, and Steinhardt (N.A.S.-N.R.C. Conference, 1958,

*Itano *et al.* (1956) note that trailing and poor separation in paper electrophoresis may be ascribable to effects arising from the preparation of the haemoglobin solution and it is known that such effects also increase rapidly with the age of the sample (Motulsky *et al.*, 1954).

p. 154) has commented that no ferrocyanide ions could ever be separated from methaemoglobin, no matter how long it was dialysed. Moreover, the authors have noted that the ultra-violet protein band minimum in methaemoglobin is partially filled in, even when the oxidation has been carried out at a pH value below 7.

Morrison and Cook (1957) claim to have separated some of these intermediates by ion exchange chromatography, and Huisman *et al.* (1958) have obtained a number of components by applying solutions of haemoglobin, which have been exposed to the action of small amounts of ferricyanide, to modified cellulose columns. Spectra of the fractions are given, but the extinction values of the methaemoglobin and oxyhaemoglobin maxima are not consistent with an explanation in terms of stepwise oxidation of the haems.* Cases where such intermediates might pass as separate haemoglobin species are not slow to suggest themselves, particularly where the oxidation process has been arrested, for instance by the transference of a sample to a cold room. Such intermediates might also be anticipated in the blood of subjects with methaemoglobinemia. Horecker (1943) found that after standing about two weeks haemoglobin solutions develop a band at 820 μ , suggesting the formation of methaemoglobin, but no corresponding decrease in CO-combining capacity is observed.

Modification of haemoglobin of the type described would lead to changes in some of the chemical and physical properties of the molecule. The effect of slightly elevated temperature and unfavourable conditions of pH and ionic strength on the number of titratable sulphhydryl groups (Benesch *et al.*, 1955; Murayama, 1957a, 1958) and the nature of the oxygen dissociation curve (Takashima, 1954, 1955; Wyman, 1948, etc.) requires no further elaboration here. Kubo (1956) has shown that somewhat denatured haemoglobin, still capable of oxygen binding, has an equilibrium curve, the shape of which is such as to suggest that one only of the four haems is affected. Such deviation from the behaviour of fresh haemolysates is exhibited by solutions which have merely been stored in the cold for a matter of a few days. Betke's experience (1957; Betke *et al.*, 1956b) that the rate of oxidation of oxyhaemoglobin with sodium nitrite depends on the means of preparation of the solution is explicable in terms of modification of

the molecule during haemolysis or subsequent treatment. It is likely also that such results could arise from a partial oxidation. The ease with which sulphhydryl groups are oxidized in haemoglobin, not only by such reagents as the ferricyanide ion (Remmer and Meyer-Wilmes, 1958) but also by atmospheric oxygen (Tsen and Tappel, 1958) is well known. Taylor (1955) has shown that the oxidation of canine haemoglobin, which is attended by the removal of two active sulphhydryl groups, gives rise to a haemoglobin having completely new physical properties, in particular increased solubility, and a changed electrophoretic mobility. The bearing that this observation could have on the minor components of normal adult haemoglobin is self-evident. Other examples of the same kind spring to mind, but the important question of the minor components of normal adult haemoglobin is of specific interest.

Minor fractions of normal adult haemoglobin were obtained by Morrison and Cook (1955b) from oxyhaemoglobin by column chromatography on ion exchange resin at pH 6.3. The minor fractions constituted 10% and 6% respectively of the total and were designated A_3 and A_2 . Rechromatography of the main peak gave no distinct heterogeneity, but the shape of the elution curve as reproduced (Morrison and Cook, 1955b, 1957) suggests that small quantities of A_3 might have reappeared. Subsequently, moreover, Morrison (N.A.S.-N.R.C. Conference, 1958, p. 167) has reported that under suitable conditions the first fraction could indeed be made to yield an extra component, which he surmises to be methaemoglobin reductase.* Three minor haemoglobin components, all fast running, were obtained on columns by Allen *et al.* (1958), but no slow component (A_2) was found. Again, Huisman *et al.* (1958) on modified cellulose columns find a fast-running component of about 10%, another of 0.5 to 2%, associated, however, with a wave of increased pH and therefore presumably spurious, and a slow component of 0.5 to 2%. (It is significant that the same authors found no increase in the proportion of slow-running component in thalassaemia major haemoglobin.) The chromatography of cord blood haemoglobins by Cook and Morrison (1956) has already been mentioned, and it must be said that the concentration of the minor component, viz., 10%, accompanied by 20% of normal Hb-A, seems too high to be acceptable. No component

*Attention should be drawn to the complex nature of the interactions occurring in mixtures of oxy- and methaemoglobin discussed by Scheler and Jung (1954), and said by them to involve the possible oxidation of sulphhydryl groups in the globin.

*The A_3 component has been studied by Kunkel and Bearn (1957) by incorporation of Fe^{59} , and appears to be a first natural degradation product of Hb-A.

of this sort has been reported by other authors, and has not been observed by the present authors on any of a large number of ion exchange columns under various conditions. Such minor components as have been reported in association with Hb-F (Prins and Huisman, 1956b; Allen *et al.*, 1958) have been fast-running ones. The abnormally high stability of Morrison and Cook's component in alkali makes it highly probable that this material is either a modified or bound pigment.

Although minor components have been observed and estimated by paper electrophoresis (see above) the only other preparative separation has been by Kunkel and his co-workers of A_2 , using starch slab electrophoresis (Kunkel and Wallenius, 1955; Kunkel *et al.*, 1957). The A_3 was distinguished only as a pointed front of the main zone, suggesting strongly that this component, if not a natural degradation intermediate (Kunkel and Bearn, 1957), is a denaturation product, resulting from adsorption effects, etc., or the haemolysis procedure. Whether the A_2 component of Morrison and Cook is identical with that of Kunkel *et al.* or indeed the components claimed by Van Fossan (1954), Roche and Derrien (1951), Roche *et al.* (1952), and other workers by various methods, is not known, and here again precise measurements of the absorption spectra, particularly in the Soret region, might give an indication as to the nature of the product. Kunkel *et al.* (1957) are uncertain about the homogeneity or otherwise of their A_2 sample because of the rapid denaturation which sets in even at 4° C. Electrophoresis of the A_2 in a supporting medium of starch block or polyvinyl chloride shows a slower component, but this may be a derivative of A_2 , presumably a product of partial denaturation. It is by no means unlikely that the minute A_2 and " A_4 " components separated by Masri *et al.* (1958) and Ceppellini *et al.* (1958) on starch from enormous haemoglobin samples are products of this kind.

Hoch (1950) found by free boundary electrophoresis that fresh haemoglobin solutions contain two small fast components. If a solution was kept for two weeks a small new peak appeared between the main peak and the slower minor component. The situation was further complicated by the work of Berry and Chanutin (1957, 1958), who were concerned with the storage of blood. These authors found that haemolysates of very fresh blood, when examined in a free boundary apparatus in 0.05 M cacodylate buffer at pH 6.5, gave two components, the minor one

comprising 25% of the total red cell extract. On standing, the proportion of this component gradually decreased, its durability varying between samples from healthy and anaemic subjects. This observation is clearly not compatible with the results of other authors mentioned above. What it does suggest, however, is that the minor component is due to a reversible modification of the Hb-A or to binding with erythrocyte constituents. That variations are due to aberrations in the number of haems per protein entity cannot be ruled out, though Beutler (1958) has shown that such molecules are not in fact synthesized, even under conditions of extreme iron deficiency.

One further debatable question must be discussed: the nature of the so-called haemoglobin M. It must first be said that the denaturation of methaemoglobin with mild reagents, such as sodium salicylate, urea and urethanes, has been carefully studied (Schlegel and Johnson, 1949; Banaschak and Jung, 1956) and the nature of the irregular denaturation curve has led Chalopin (1953) to postulate the heterogeneity of methaemoglobin. Steinhardt and Zaiser (1955), on the contrary, believe that this behaviour arises from steps in the denaturation process,* and Derrien and Reynaud (1955) find that electrophoretic heterogeneity in solutions containing Hb-F disappears on oxidation. Chalopin (1958) has observed that sodium salicylate denatures methaemoglobin without precipitation, if the concentration of the denaturing agent is high. Schlegel and Johnson (1949) have followed the process spectrophotometrically and have also found that it is not accompanied by a volume change.

Since this is usually considered one of the characteristics of the denaturation of proteins, the methaemoglobin should be regarded as "modified" rather than denatured. Chalopin (1958) moreover gives reasons for supposing that a wide range of products, in various stages of attack, is present. It is likely in any case that the reduction products of such a methaemoglobin would be scarcely distinguishable from native haemoglobin. Banaschak and Jung (1956) and Schlegel and Johnson (1949) give absorption spectra in the visible region of intermediate stages in the reaction of methaemoglobin with sodium salicylate

*The nature of the reversible and irreversible stages in denaturation is as yet obscure. The early stages appear to involve an unfolding or swelling of the peptide chains, followed by the breakage of S-S bridges and possibly their subsequent regeneration in different positions (Stauff, 1956). It seems possible that ultra-violet spectrophotometric examination of the globin bands at various stages in the denaturation process may furnish information (Nakajima, Nakanishi, Kaminaga, Araya, and Kikuchi, 1955).

and urethanes, and some of these bear a marked resemblance to the visible spectra of methaemoglobin M shown by Hörlein and Weber (1948), Kiese *et al.* (1956), and Heck and Wolf (1958). Both the latter groups of authors have found that the spectroscopic deviations do not extend to any of the haemoglobin M derivatives, and that the abnormality lies in the globin and not in the haem. This is interesting, but does not exclude the possibility that the abnormal methaemoglobin is due not to a genetic variant of the haemoglobin but merely to a modification of the protein by erythrocyte constituents. This could occur by attack on the haemoglobin with methaemoglobin formation in the first or a subsequent stage. The latter is evidently the more likely alternative, since Kiese *et al.* find that the rate of oxidation of haemoglobin of a subject suffering from this form of methaemoglobinaemia is almost twice as great as that of normal adult haemoglobin (with consequent overloading of the methaemoglobin reductase system in the erythrocyte). Also Gerald *et al.* (1957) claim to have isolated by starch block electrophoresis a precursor haemoglobin, which when oxidized gives the spectrum of the abnormal methaemoglobin M. The resolution is not good, however, and the results are vitiated by contamination of the haemoglobin with the incompletely separated methaemoglobin. (A similar separation has been reported on paper by Pisciotta, Ebbe, and Hinz (1957), who find a slow-running haemoglobin to be present. Their sample contained 11% of alkali-resistant pigment, but no mention is made of the appearance of Hb-F on paper. The authors conclude that the slow-

running material, Hb-M, is incapable of carrying oxygen. The argument, however, is somewhat obscure and in any case runs counter to the results of Kiese *et al.* (1956), who find that the oxygen dissociation behaviour of their specimen is normal.) More recently Gerald (1958) has described in detail the spectroscopic and electrophoretic characteristics of an Hb-M specimen. It was found in particular that a clear separation on starch slab could be obtained of two components in the haemolysate after ferricyanide oxidation. One of these exhibited all the normal characteristics of methaemoglobin, but the other showed an extraordinary anomalous reaction to sodium cyanide. On treatment with small amounts of this reagent a double Soret peak was observed, and this was eliminated, with simultaneous formation of the characteristic cyanmethaemoglobin spectrum, only on addition of a large excess of cyanide ion. This suggests that an unusual type of linkage is present in at least one of the haem groups of the molecule, in which a bound component is displaced only with difficulty by cyanide. Such an effect could arise from an extra link to the globin, arising from partial denaturation or unfolding of the protein, or to an extraneous substance. This situation is strikingly reminiscent of the supposed equilibrium between bonded globin and cyanide ion described by Tsushima (1956).

It would be of interest in connexion with the Hb-M problem in general to seek a pathological denaturing agency within the erythrocyte.

[The second part, together with a list of references, will appear in the March issue.]