ELECTROPHORETIC BEHAVIOUR OF HAEMOGLOBINS IN AGAR GEL

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INTRODUCTION

Starch and agar gels are now widely used as supporting media for the zone electrophoresis of proteins. Agar gel below neutral pH was found by ROBINSON *et al.*[1] to be of particular value for the study of human haemoglobin variants. The patterns obtained exhibit certain features, however, which suggest that the mechanism of separation is not simply electrophoretic, but that other factors are involved. Agar gel electrophoresis of proteins at conventional pH (8–9) has been widely used, and gives results which are often superior to those obtained on paper[2–4], for instance, with serum proteins. In this pH range the separation of haemoglobins is comparable with their behaviour on paper[5–7], in particular with respect to the sequence of migration, which reflects the isoelectric points. Thus, Hb-S*, Hb-C, etc. separate on the cathodic side of Hb-A, and Hb-F is not resolved from the latter. If, however, the pH is reduced to 6–6.5, Hb-F is widely separated from Hb-A[1] and Hb-S and Hb-C separate clearly on the anodic side. In addition Hb-D—which on paper and other electrophoretic media is indistinguishable from Hb-S—does not separate from Hb-A. Other features are the characteristic curvature of the pigment zones, other than Hb-F, and the fact that only this haemoglobin appears to have a constant mobility.

EXPERIMENTAL

The following procedure has been used in the present work for the routine separation of haemoglobins. A 1% gel of Difco Bacto Agar in *M*/2 citrate buffer (pH 6.2), is prepared by boiling and poured on a thin glass plate (10 in. × 4 in.) to give a 1 mm layer. Strips of Whatman 3 MM filter paper, slightly narrower than 1 mm, are impregnated with haemolysates, lightly blotted and inserted into slits made with a razor blade, about 3 in.–4 in. from one end of the gel. The plate rests on the rims of plastic boxes containing buffer solution, with which contact is made by filter paper bridges resting on the surface of the gel. The latter is protected against evaporation

* Abbreviations: Hb-A, human normal adult haemoglobin; Hb-F, human foetal haemoglobin; Hb-S, Hb-C etc. are the various genetically-determined variants of human haemoglobin.

with a sheet of polythene film lightly smeared with paraffin oil. Contact between the buffer reservoirs and reversible electrodes is made with strips of plastic sponge soaked in buffer. Optimal separation is achieved in *ca.* 4 h, with a potential of 40 V across the gel (total applied voltage 200 V), with a current of 20 mA. After completion of the electrophoresis the pattern is photographed by contact, and may then be stained with a protein or haem-specific stain. For the former, the requisite part of the gel is transferred to a smaller glass plate, the protein zones fixed by immersion in methanol–acetic acid mixture for some minutes, and the gel allowed to dry overnight in air. It is then stained with amidoblack or azocarmine by the procedure of URIEL AND GRABAR[8]. Benzidine staining is best carried out immediately after electrophoresis and the stabilised reagent of SWARUP *et al.*[9] gives excellent results. The direct spectroscopic examination of separated zones of native haemoglobin in agar gel has already been described[10].

The unusual zone shapes (Fig. 1) preclude the estimation of haemoglobin components by simple densitometry, but reliable results can be obtained by dye-elution. After staining and differentiation with a glycerol-containing reagent[8] the zones can be excised with a razor blade, peeled from the plate, the dye eluted in a known volume of *N*/50 aqueous alkali (*e.g.* 4 ml) and estimated spectrophotometrically (at 620 mμ for amidoblack).

RESULTS

Human haemoglobin variants

Fig. 1 shows typical separations of several human haemoglobins. The curvature of all the zones, other than that of Hb-F, is characteristic. It is also apparent that the relative mobilities of the various species cannot be compared in different samples. The haemoglobin variants, the behaviour of which in agar gel electrophoresis at low

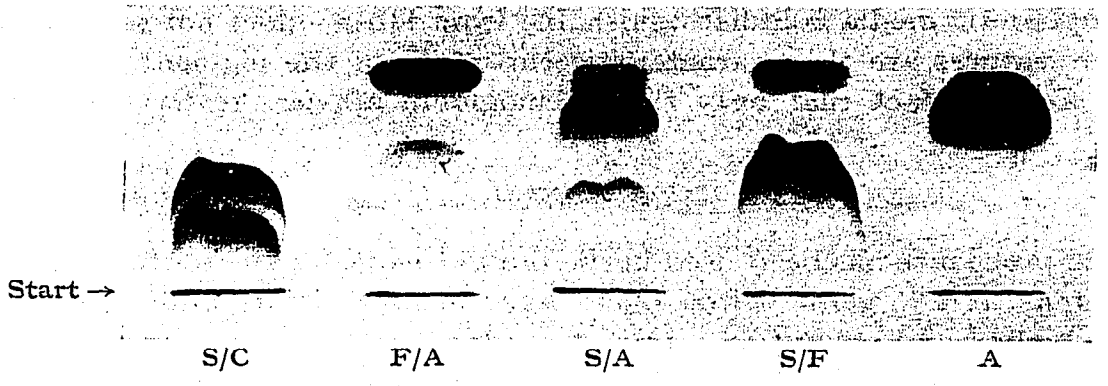

Fig. 1. Typical agar gel electrophoresis patterns of human haemoglobin mixtures; *M*/20 citrate buffer, pH 6.2; contact photograph of unstained zones.

pH has previously been described, are Hb-S, Hb-C[1,11] (all slower than Hb-A) and the following, which behave identically with Hb-A: Hb-D[1,12], Hb-E[13], Hb-I, Hb-J, "Chernoff-N", Hopkins-I, Hopkins-II, Lepore[12]. The relative mobilities of haemoglobins in agar electrophoresis at pH 6, compared with conventional zone and free-

boundary electrophoresis, are as indicated in Fig. 2. Several samples containing Hb-H were examined, and this variant was found to migrate more slowly than Hb-A, but unlike the other variants, with a straight front (Fig. 3).

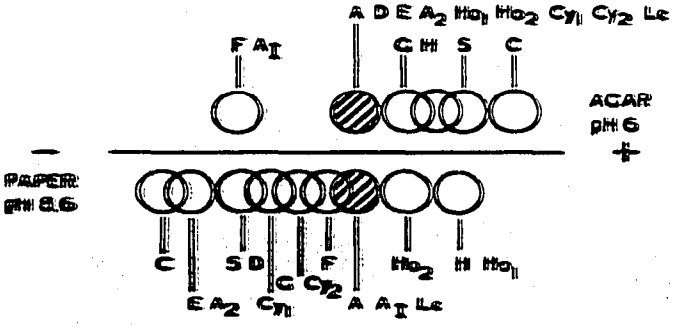

Fig. 2. Migration sequences of human haemoglobins in agar gel (*ca.* pH 6) and paper (pH 8.6) electrophoresis.

All samples of Hb-A also show a minor component (Hb-A_I) running in the position associated with Hb-F, from which, however, it has been distinguished[13], and a further small component[12,13] which appears to arise from ageing of the haemolysate. Other minor components of Hb-A are indistinguishable from the main component[13].

Experimental variables

(*a*) *Type of agar.* Unlike agar electrophoresis at high pH, the results with haemoglobins below neutrality are critically dependent on the agar used. Of seven commercial preparations tested, Difco Bacto Agar and B.D.H. Japanese agar give separations

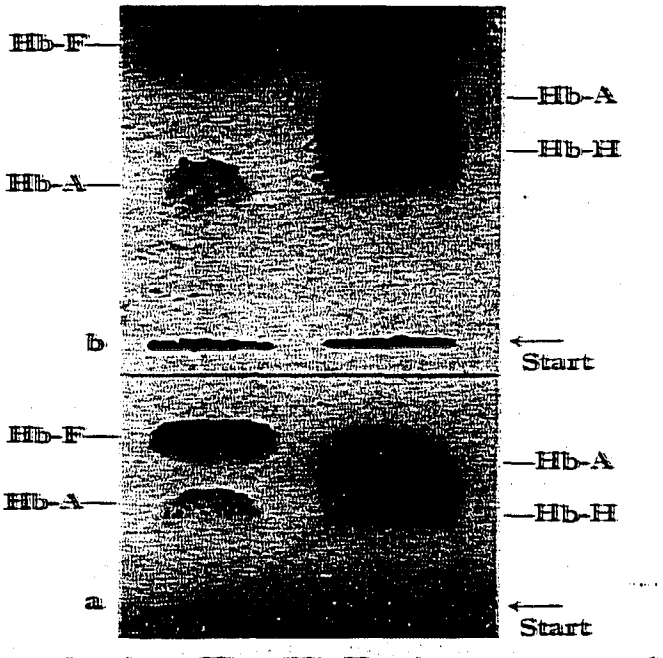

Fig. 3. Agar gel electrophoresis of an Hb-A/Hb-H mixture, compared with an Hb-F/Hb-A mixture; (a) 4 h, (b) 6 h migration.

of the kind shown in Fig. 1 (the former being the more satisfactory), while the remainder gave no separation whatever. Fig. 4 shows typical results for the same samples as used in Fig. 1, examined in Difco Noble Agar (a purified form of Bacto Agar) and Light's Ionagar II respectively. Different batches of Difco Bacto Agar all gave satisfactory results, and a sample purified by the procedure described by BUSSARD AND PERRIN[14] showed unchanged behaviour.

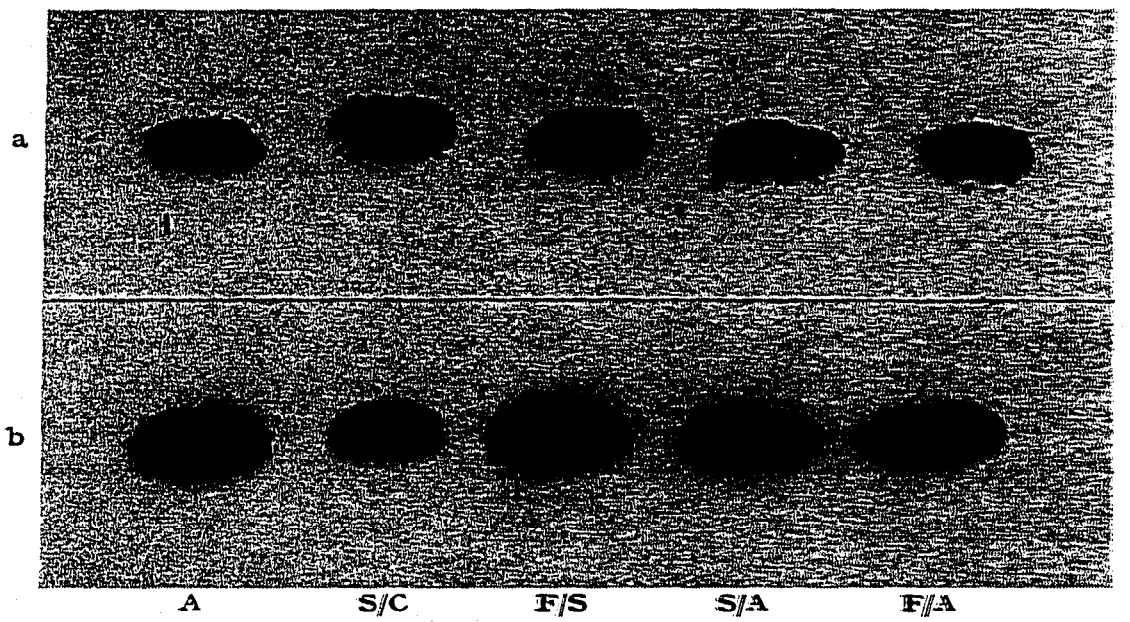

Fig. 4. Agar gel electrophoresis of human haemoglobin mixtures in 1% gels of Difco Noble Agar and Light's Ionagar II, showing absence of separation.

(*b*) *Thickness.* It has been found important to restrict the gel thickness to *ca.* 1 mm in order to minimize heating effects. In thicker layers spurious zones appear, many of which can be suppressed by incorporation of cyanide (*ca.* *M*/100) in the buffer, and may therefore be presumed to be methaemoglobin and intermediate oxidation stages[15].

(*c*) *Voltage across gel.* Any appreciable increase in the potential gradient above about 2 V/cm leads to rapid heating, again with formation of spurious zones. The temperature at the surface of a 1 mm layer under the standard conditions is *ca.* 27° and must therefore be considerably higher at the centre of a thicker layer. Since on relatively brief exposure, even to 35°, haemoglobin undergoes considerable changes in properties, including electrophoretic mobility[16], the joint restrictions on gel thickness and working voltage are of great importance.

(*d*) *Duration of electrophoresis.* For Hb-F and the minor component Hb-A_1 migration distance in the gel is directly proportional to time (see below). With haemolysates of the usual concentration (*ca.* 10–15%) the distribution of zones is optimal after about 4 h. With the slow-moving haemoglobins migration ceases after about this period, and in some cases the zone actually begins to move back towards the cathode (Fig. 5).

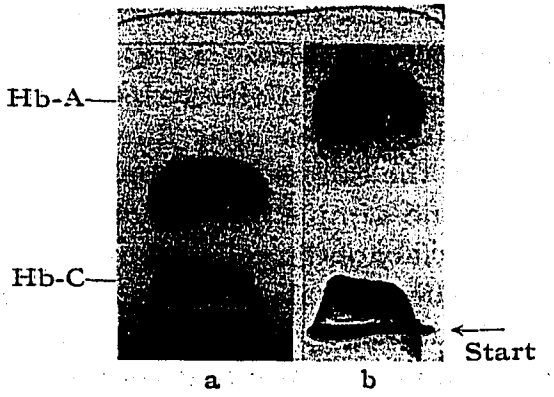

Fig. 5. Agar gel electrophoresis of an Hb-A/Hb-C mixture, photographed (a) 4 and (b) 6 h after start of experiment.

(e) *Condition of samples.* The mobility of a pigment in the gel depends on its concentration, except in the case of Hb-F and Hb-A_1. Fig. 6 shows the electrophoresis of serial dilutions of an Hb-A/Hb-F mixture. Whereas the mobility of the Hb-F remains constant, the Hb-A component is progressively retarded with dilution. This effect occurs only below a limiting concentration and it is therefore desirable to use concentrated haemolysates. Retardation cannot be avoided, however, when a component is present in low proportion, such that its own partial concentration is low. The discrepancy between the mobility of Hb-A in a normal adult haemolysate and in cord blood haemoglobin (*cf.* Fig. 1) is explicable in these terms, and must be regarded as a fundamental drawback of the method.

Equilibration of the samples is unimportant, as prior dialysis against buffer has no effect on the separations or the zone shapes.

Fig. 6. Agar gel electrophoresis of serial dilutions of: (a) an Hb-A/Hb-F mixture; (b) an Hb-A/Hb-C mixture; benzidine stain. Note fast-running minor component (Hb-A_1).

(f) *Application of samples.* The possibility was considered that the zone curvature and back-streaming arise from the presence of the filter-paper strips in the sample slits, or the slits themselves. Removal of the strips after the pigment has entered the gel, and sealing the slit with molten agar do not affect the results, however. No improvement is found when the samples are applied differently, *e.g.* by pipetting a just molten mixture of agar and sample into a slot, or by impregnation of broader filter paper strips lying on the surface of the gel, as proposed by MARDER AND CONLEY[12].

(g) *Buffers.* The nature of the separation depends on pH, ionic strength and the anionic species present. The ionic strength is not very critical, but any considerable increase leads to excessive heating under the requisite potential gradient. At a citrate ion concentration of less than *ca.* *M*/30 zone curvature and back-streaming become very pronounced.

A number of buffers cause precipitation of haemoglobin near the start line. These include pyridine acetate, phthalate, and, to some extent, cacodylate. With other buffers, back-streaming and the dependence of mobility on pigment concentration are very marked, *e.g.* phosphate and malcate. In an unbuffered system (*M*/20 sodium chloride), where the local pH is presumably determined by the protein, reasonable separations are achieved.

Good results, comparable with those in citrate, are achieved by the use of *M*/25 ethylenediaminetetraacetic acid (EDTA) buffer, and indeed the zones are somewhat sharper (Fig. 7). The effects of variation in pH are discussed below.

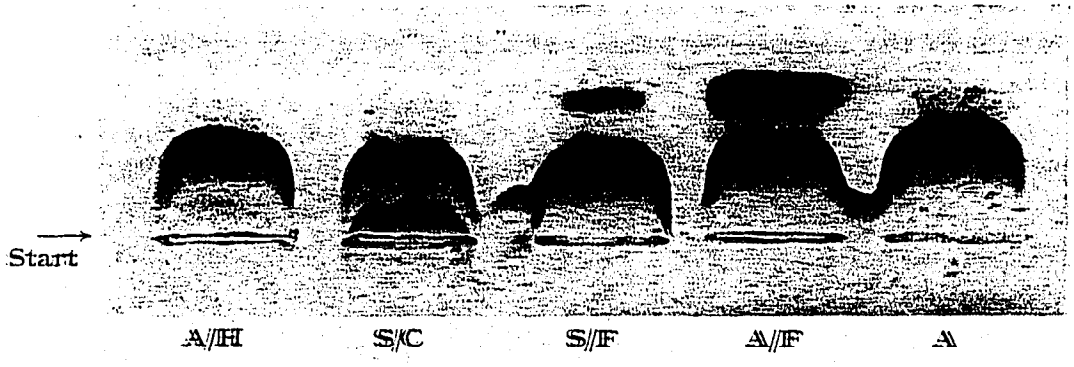

Fig. 7. Agar gel electrophoresis patterns of human haemoglobins in *M*/25 EDTA buffer, pH 6.4.

MECHANISM OF SEPARATION OF HAEMOGLOBINS

Sequence of migration

In electrophoresis in other supporting media, including agar gel at higher pH, the separation of human haemoglobins is governed by differences in their overall charge. In a group of such closely related species a simple relationship of this kind is to be expected, and the degree of separation of any two pigments is in fact closely related to their relative mobilities in free-boundary electrophoresis[16]. The sequence of separation of a series of haemoglobins:

H F A S C

will be independent of the direction of migration (except possibly over an extreme pH range, insofar as their mobility/pH curves may not be parallel) and will represent the order of isoelectric points. In agar electrophoresis under the stated conditions the sequence is:

C S H A F

This alteration indicates that the separation is not primarily dependent on the charge, but on factors which must reflect some other features of the protein structure. Since the above species have isoelectric points between the approximate limits[16] of pH 5.6 (Hb-H) and 7.3 (Hb-C), these factors must be of predominant importance.

Specificity for haemoglobins

The mechanism in question evidently applies only to the haemoglobins. When serum proteins are run in agar gel under the same conditions (pH 6–6.5) the separation is somewhat similar to that obtained on paper, and certainly no better; the characteristic curved fronts are not observed.

Any form of "molecular sieve" mechanism, such as is believed to operate in starch gel electrophoresis[17] can be excluded, as GORDON *et al.*[2] found that retardation with increasing agar concentration occurred only for proteins of very large molecular weight. Furthermore the behaviour of serum proteins in agar gel suggests that only a conventional electrophoretic process is operating. In any case the identical molecular weights, and presumably closely similar shapes, of the haemoglobin variants would render a molecular sieve mechanism irrelevant.

Similarity to ion-exchange columns

Some remarkable similarities exist between the migration sequences of haemoglobins in agar gel electrophoresis and their order of elution from ion-exchange columns at the same pH[18]. This, in increasing order of R_F value is:

C D S E G A F H

compared with the migration sequence

C S H G A D E F

for the same species on agar.

Hb-F is scarcely adsorbed on the resin and Hb-E moves only slightly slower than Hb-A[19]; both are therefore quite similar on columns and in agar gel by contrast with their normal electrophoretic behaviour. The behaviour of Hb-H can presumably be explained by its outstandingly low isoelectric point. The position of Hb-D is a real anomaly. It seems possible, however, that the different forms of this variant[20], though electrophoretically identical on paper, might differ in agar electrophoresis and column chromatography.

A situation may now be envisaged in which the haemoglobins are "eluted" in the cathodic direction by the electroendosmotic buffer flow, Hb-F being essentially unadsorbed. Haemoglobins, A, S and C will be desorbed in the same order as on ion-exchange columns. The "R_F" value of Hb-F is less than unity, however, (*v.i.*) and this can be explained in terms of its retardation by the superimposed field. From available isoelectric point data[16] most of the haemoglobins would be expected to be positively charged at the working pH, but PRINS[21] states that their isoelectric points are lower by two pH units in citrate (μ 0.1) compared with phosphate. Differences in the same sense were earlier observed for horse haemoglobin[22]. The field will then be opposing the "elution" towards the cathode. This situation is entirely consistent with the behaviour of the pigments at still lower pH (*v.i.*).

Dependance of adsorption on pH

By analogy with ion-exchange columns[23] the degree of adsorption should decrease with increasing pH, and if the slowest-moving zones in agar are the most strongly adsorbed, they should be successively desorbed by progressive increases in buffer pH.

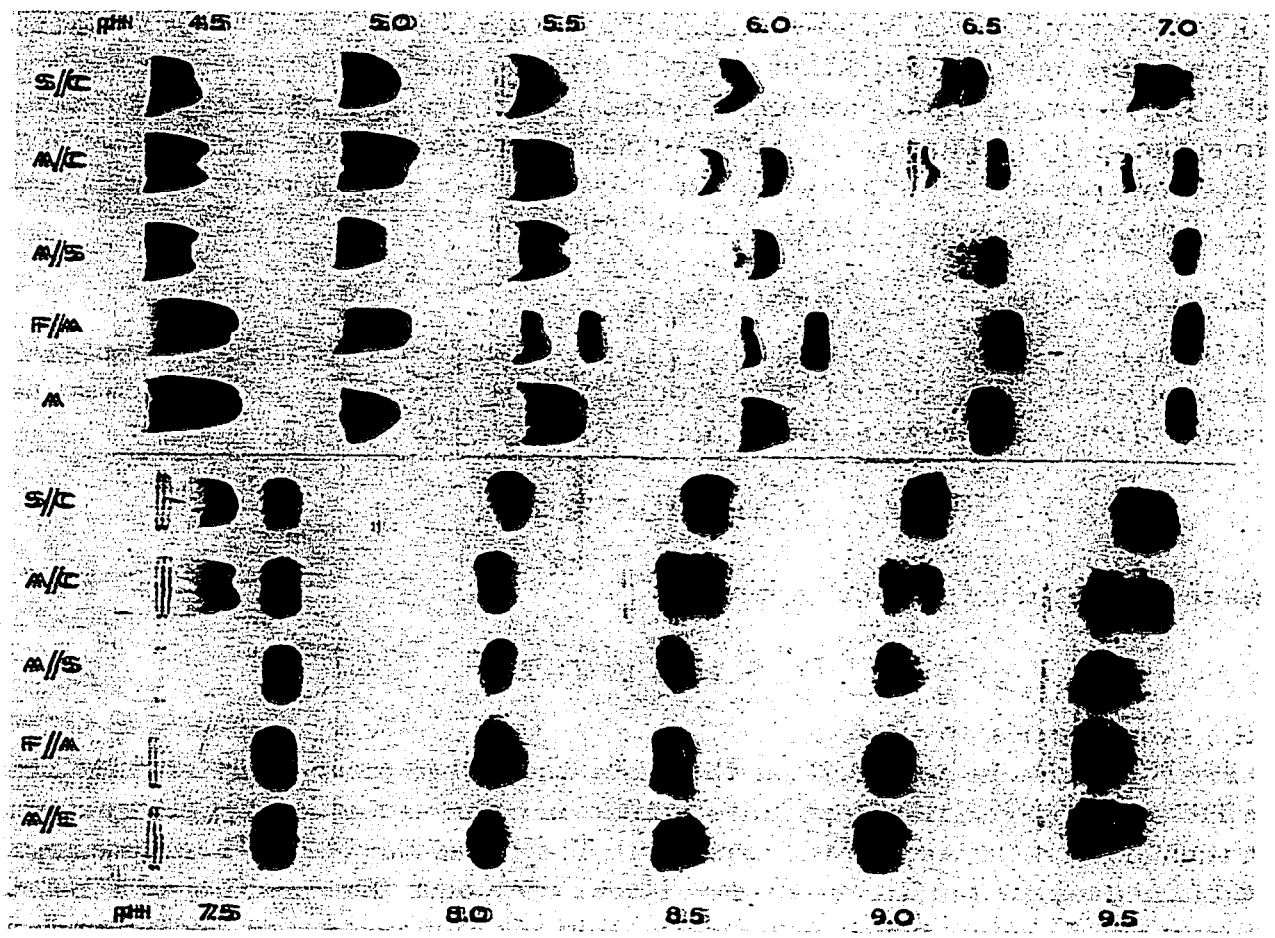

Fig. 8. Effect of pH variation on separation of human haemoglobins in Difco Bacto Agar gel, using acetate, citrate, triethanolamine or barbital buffers of constant ionic strength. An Hb-A/Hb-E mixture was used instead of Hb-A over the pH range 8.0–9.5.

Examination of a typical agar electrophoresis pattern (*e.g.* Fig. 1; see also MARDER AND CONLEY[12]) shows that zone curvature and trailing is most marked with the slowest-moving haemoglobins. The desorption which accompanies an increase in pH should eliminate the manifestations of adsorption. The results of agar gel electrophoresis over the pH range 4.5–9.0 are shown in Fig. 8, for buffers of constant ionic strength. At the lowest pH severe trailing, with probable denaturation, is occurring, but in the range 5.0–5.5 separations in the above-mentioned sequence are observed. The Hb-F zone, however, has the curved shape of the other haemoglobins and may therefore be considered to be adsorbed. At pH 6.0 the Hb-F zone is straight, and the Hb-A zone is the next to lose its curved shape with further increase in pH; by pH 7.0 it is completely straight and resembles that of Hb-F. Moreover, the mechanism responsible for the separation of these two pigments is now suppressed, and they migrate in mixtures as a single zone. Hb-S is next desorbed and by pH 7.5 it too has a straight zone shape and is not resolved from Hb-A. Hb-C is the last to retain a curved front, and this is only entirely lost by pH 8.0. At this point there is little separation of any of the variants. With still further increase in pH, the usual region for the electrophoretic separation of approximately neutral proteins is reached, and agar gel is then operating as a normal supporting medium. The samples shown in Fig. 8 include a specimen of Hb-E trait haemoglobin, and this variant, which is unresolved at the lower pH value, is now separated. The resolution of Hb-C is clear, and it will be noted that the relative positions of Hb-A and Hb-C with respect to polarity are reversed, compared with the lower pH. Thus the electrophoretic mechanism at high pH is conventional and depends on charge differences. Because of the large magnitude of the electroendosmosis the displacement is always cathodic.

Chromatography in agar gel

If the mechanism of fractionation of haemoglobins in agar gel at low pH is adsorptive, it should be possible to dispense with the potential gradient if a liquid flow can be maintained.

Accordingly a mixture of Hb-A and Hb-F (*ca.* 10 mg) was rapidly mixed with some just-molten agar gel and poured on the surface of a column of agar gel (10 cm × 1 cm diameter). After this had set, another layer of just-molten agar was added, and the column eluted with *M*/20 citrate buffer. Elution was slow (less than 1 ml in 24 h) but the haemoglobin migrated down the column. The first eluate fractions were yellow due to an impurity in the agar. The pigment was eluted after some days at 4° as a moderately concentrated solution. No separation of zones on the column was observed, possibly because of diffusion, but examination of the first pigment fraction[25] by the moving-plate spectrograph[24] showed it to be almost pure Hb-F, indicating that the adsorption mechanism was operative.

Dilution effect and adsorption isotherm

An explanation of the dilution effect (Fig. 6) is now evident. It is known[25] that the adsorption of proteins often follows the empirical Freundlich isotherm: $c_a \propto c_s^{n/m}$

where c_a and c_s are the respective concentrations of the adsorbate in the adsorbent and the continuous phase and n a constant (the adsorption constant). This behaviour is a consequence of the progressive occupation of sites on the adsorbent as the adsorbate concentration increases. An asymptotic maximum is thus approached. That such an effect is operating here is shown by the plot (Fig. 9) of the concentration of Hb-A applied to the gel (in arbitrary units) *vs.* migration distance in a constant time; the latter should be simply related to the proportion of unadsorbed pigment.

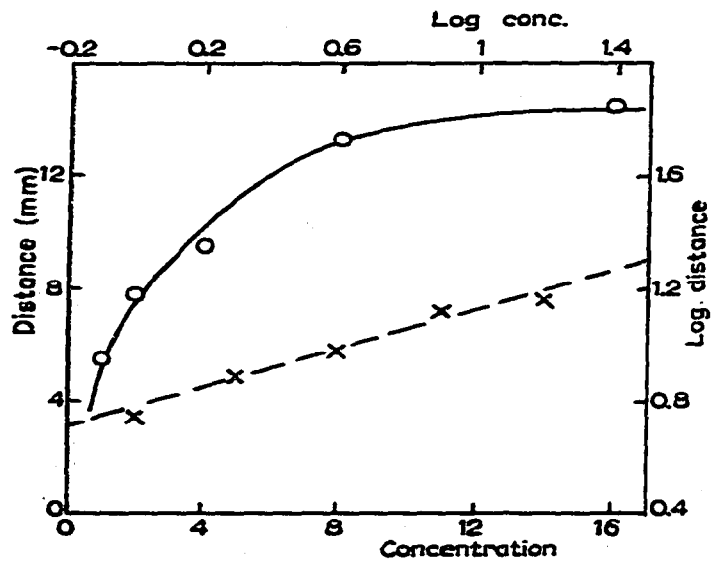

Fig. 9. Direct and logarithmic plots of migration distance (mm) of Hb-A zones *vs.* pigment concentration (arbitrary units).

A marked resemblance to a Freundlich isotherm is evident. The logarithmic plot is linear and its slope is 0.36 ($n = 2.8$), which is comparable with values reported for various adsorbing systems involving proteins[26,27]. The increased back-streaming and decreased mobility, which accompany a reduction in ionic strength, are also in accord with the results of adsorption experiments on the majority of proteins, whereby adsorption is repressed by high salt concentration[25].

Retardation of zones with time

The progressive retardation of zones other than that of Hb-F culminating, in the slowest-moving electrophoresis variants, in reversal of migration, might be thought to be due to a change in electroendosmotic flow with time. To test this possibility, hydrogen peroxide was used as an uncharged marker in an electrophoresis at pH 6.2. At intervals filter-paper prints of the gel surface were taken and stained with ammoniacal silver hydroxide to detect the marker, followed by amidoblack to locate the haemoglobins. Fig. 10 is a plot of migration of marker and Hb-F *vs.* time, showing that over the duration of the experiment the electroendosmotic flow remained constant at 1.3×10^{-4} cm/sec.

The only possible explanation of the retardation, therefore, is the effective

decrease in concentration of protein in the gel as the zones diffuse. Because of the dilution effect described above the degree of adsorption of the pigment then increases, with consequent progressive retardation of the zones.

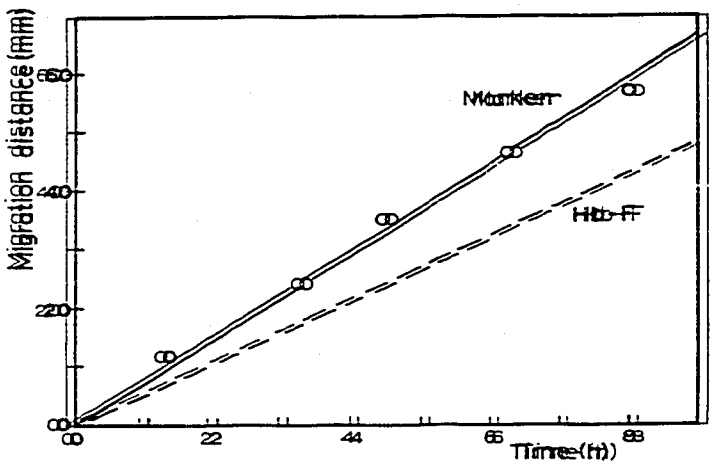

Fig. 10. Plot of migration distance (mm) *vs.* time (h) of uncharged marker (hydrogen peroxide) compared with Hb-F zone.

Reversal of strongly adsorbed zones

The actual reversal of the slowest-moving zones is less easily explicable. It might, however, be expected that the more dilute the protein the greater its degree of anion-binding, on a stoichiometric basis. A point might thus be reached where the additional negative charge so acquired would be sufficient to cause anodic migration. It seems likely that strong specific binding, of the kind known to occur between some proteins and small anions[28], is occurring here. It may also be envisaged that the mechanisms of desorption of proteins under a potential gradient on the one hand and by a buffer flow on the other, may differ. A dilution effect has also been observed for human serum albumin, its mobility changing with concentration both in paper[29] and free-boundary[30] electrophoresis.

The different results obtained with various buffers may also be explicable in terms of anion binding. Ions of high polarisability should be especially strongly bound, but the addition of thiocyanate or cyanide does not affect the electrophoresis patterns in agar. It appears to be generally true, however, that the greater the valency of the ion, the greater its interaction with proteins[31]. The superiority of the results obtained with citrate and EDTA buffers may be due to this effect. In particular, citrate ion, which at pH 6.0–6.5 is (unlike phosphate) at the upper limit of its buffering range, carries a high charge and might be expected to interact strongly with haemoglobins.

Zone curvature

The remaining phenomenon in agar gel electrophoresis to be explained is the distinctive zone curvature which accompanies adsorption. It has already been shown

that this is not caused by the sample slit nor is it in any way dependent on the manner in which the sample is applied. It must therefore arise directly from the influence of the protein itself on the ionic strength and pH in its vicinity, and is a zone boundary effect. An analogy with the behaviour of a protein on a cation-exchange column may again be drawn: as the protein zone moves, cations are displaced with a measurable heat of desorption[32] and a front of increased ionic strength precedes the zone through the column, accompanied by a readily detectable pH ripple (see *e.g.* GUMMER *et al.*[33]). At the same time an ionic strength and pH discontinuity must develop behind the protein zone. A similar process may be envisaged in agar gel. Here, however, an extra dimension has to be considered, since the zone also has boundaries parallel to the direction of migration. The ionic strength discontinuity at the zone boundaries should be associated with an increase in adsorption of the protein, resulting in retardation of protein transport by the buffer flow, *i.e.* zone curvature and "back-streaming".

Differences between agar preparations

It remains to consider in what structural respect Difco Bacto and BDH Japanese agars may differ from other agar preparations which are not effective as supporting media under the standard conditions. The difference seems likely to reside in variations in the degree of ionization, number, or steric disposition of the acidic groups, or a combination of these factors. If the difference is related to the total electrostatic charge in the gel structures, there should be corresponding differences in zeta-potential, which will be reflected in the magnitudes of the electroendosmosis. An indication that this might be so came from a comparison of serum protein patterns after electrophoresis in the same experiment in Difco Bacto Agar and in Light's Ionagar II gels of the same concentration. Fig. 11 shows the striking differences between the positions of the zones, which must be caused by differences in electroendosmosis.

Gels containing identical concentrations of the seven available types of agar in the same buffer were loaded with electroendosmosis markers (hydrogen peroxide, glucose or dextran). At hourly intervals during electrophoresis filter-paper prints of

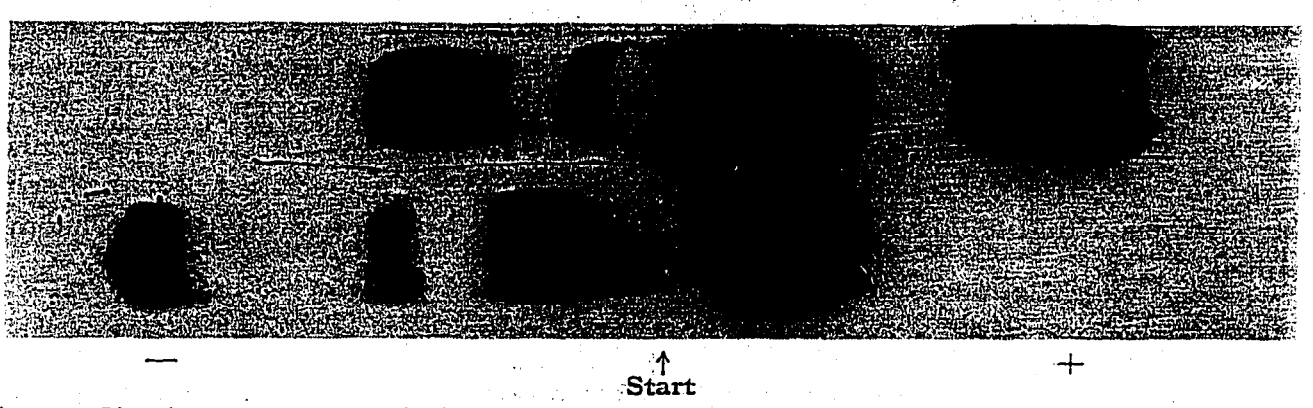

Fig. 11. Simultaneous agar-gel electrophoresis of human serum in (a) Light's Ionagar III and (b) Difco Bacto Agar; 1.25 % gels; barbital buffer, pH 8.6; azocarmine stain.

the gel surfaces were taken and stained with ammoniacal silver hydroxide for hydrogen peroxide, or with aniline hydrogen phthalate[34] for glucose. Dextran was often directly visible as a zone of greater transparency. These experiments were carried out with *M*/20 citrate buffer (pH 6.2) and barbital buffer (pH 8.6, μ 0.03), and plots of marker migration distance *vs.* time in all cases gave good straight lines. The electroendosmotic flow rates are listed in Table I, from which it is clear that the values for the two effective types of agar are greater by a factor of two than those for the remainder, with only Difco Noble Agar giving an intermediate value.

TABLE I

Agar	Electroendosmotic flow rate (cm/sec/V/cm × 10^4)		Electrophoretic performance with haemoglobins
	Barbital pH 8.6, μ 0.03	Citrate pH 6.2, *M*/20	
Light's Ionagar III	1.5	0.53	— —
Oxo	1.5	0.53	— —
Light's New Zealand	1.5	0.49	— —
Gurr's Bacteriological	1.65	—	— —
Difco Noble	2.1	0.75	—
Difco Bacto	3.05	1.3	+ + +
BDH Japanese	3.1	1.2	+ +

Thus the effective gels presumably have a higher effective charge density. The responsible groups may be either polysaccharide carboxyl or sulphate[35], of which the former should be titratable in an accessible pH range. Titrations on suspensions of agars showed no acidic or basic dissociations over the pH range 5–10. The charged groups responsible for the differences in electroendosmosis are therefore not titrated in this range, and it seems likely that they are sulphate groups.

If the concentration of charged groups in a Difco Noble Agar gel was increased by making the agar concentration 3%, normal electrophoretic patterns were obtained. With the other types of agar a concentration of 5% is necessary for any separations, but the results remain poor. With Difco Bacto Agar, on the other hand, its concentration could be halved by admixture with an ineffective agar, without appreciable effect on its performance.

Relation of adsorption to haemoglobin structure

From an analysis of the ion-exchange chromatography of haem proteins, BOARDMAN AND PARTRIDGE[23] concluded that in their working pH range (below 7) adsorption did not arise from simple Coulombic forces, since the haemoglobin molecule carries little if any negative charge under these conditions, but rather from short-range interactions of a hydrogen-bonding character between undissociated carboxyl groups on the resin and acceptor groups on the protein. The degree of adsorption is thus likely to be largely dependent on the steric disposition of charge on the protein

molecule. Another treatment[36] indicates that adsorption on ion-exchange resins is mainly governed by the distances separating the carboxyl and amino groups of the protein.

The entirely different behaviour of Hb-F from Hb-A in agar gel and other adsorptive systems suggests, if their α-polypeptide chains are identical[37], that the β^A-chains contain the structural elements responsible for adsorption, whereas the α^A- and the γ^F-chains do not. The reported structure[38] of Hb-H, *viz.* β^A, is not in these terms in accord with its low adsorption. The different extent of adsorption of haemoglobins A, D and E from haemoglobins G, S and C, and these latter from each other, should be reconcilable with their known structural differences. These may be summarised in terms of the one varying amino acid unit in each β-chain, *viz.* Hb-A[39], glutamic acid; Hb-S[39], valine-; Hb-C[39], lysine-; Hb-G[40], glycine-; Hb-E[41], lysine- (for a different glutamic acid residue).

In the case of Hb-S the loss of the negative groups (carboxyls of glutamic acid, when replaced by valine) is responsible for a large increase in adsorption, and the same situation obtains in Hb-G. In Hb-C, with lysine replacing glutamic acid, the degree of adsorption increases still further. Thus the carboxylate group presumably opposes interaction with the negatively charged groups in the agar, whereas the addition of a further amino group (present as NH_3^+) promotes the interaction. A similar substitution occurs in Hb-E, but with no changes in the adsorptive behaviour. The groups involved in the substitutions in haemoglobins A, S, C and G must therefore be near the surface of the molecule, whereas the group responsible for the difference between Hb-A and Hb-E (and presumably Hb-D and other variants not separating on agar gel) must be remote from it. These results are consistent with the position near the N-terminus of the β-chain, which the aberrant residues in haemoglobins S, C and G are believed to occupy[39,40], by contrast with Hb-E, in which the difference lies in another part of the chain[41].

CONCLUSIONS

It would appear that agar gel electrophoresis under the conditions discussed above might have applications beyond its evident analytical use. The simple reasoning given above suggests that it could, when used in conjunction with conventional electrophoretic techniques, provide further correlations with structural studies. Certainly, as indicated by Figs. 8 and 9, it offers a method of greater interest for adsorption studies than the use of, for instance, alumina[42], which like most media of this kind, is essentially a denaturing agent[43]. The adsorption isotherms may be of value for the characterisation of individual haemoglobin variants.

SUMMARY

Experimental variables in the electrophoresis of human haemoglobins in agar gel have been investigated. The migration of haemoglobins in this medium below neu-

trality, including the separation of the adult and foetal pigments, is explicable in terms of an adsorptive mechanism. Differences in the behaviour of some haemoglobin variants in agar gel electrophoresis have been tentatively correlated with their known structural differences.

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