Dissociation of Human Haemoglobin at Low pH

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A number of mammalian haemoglobins dissociate under certain conditions, such as low protein, high salt or high urea concentrations (see review by Wyman, 1948). Svedberg & Pedersen (1940) claimed to have found evidence for the dissociation of human haemoglobin with dilution by observing a fall in the sedimentation coefficient at protein concentrations below 0·8% (w/v)* However, Kegeles & Gutter (1951) showed, on the contrary, that down to concentrations of 0·1% the sedimentation coefficient of human haemoglobin, at pH 7·07, rises linearly with dilution. Moore & Reiner (1944) reported that human haemoglobin separates—partially at pH 4, completely at pH 2·5—into two electrophoretic components with sedimentation coefficients of 2·5 and 2·1 respectively. These values, however, are so low, especially compared with the value, reported by these authors, of 5·5 at pH 7·5, as to suggest that dissociation, if present, must have been accompanied by a considerable degree of denaturation. This conclusion is supported by the present authors' findings (unpublished) of two electrophoretic components at pH 7·47 and 9·15 in a sample of haemoglobin prepared from old blood from a blood-bank, and by evidence, presented below, that denaturation occurs rapidly at pH values below 3·5–4·0.

Some studies, in this laboratory, on human carboxyhaemoglobin disclosed a lowered sedimentation coefficient at pH 5·06, accompanied by a raised diffusion coefficient. This finding led to the investigations, described here, on the effects of low pH in producing dissociation of human carboxyhaemoglobin and on the extent to which factors such as protein concentration, ionic strength, nature of the buffer, and the presence of copper influence this dissociation. Dissociation was studied mainly by observing changes of the sedimentation coefficient, but in addition some diffusion coefficient determinations were carried out in order to ascertain the changes in molecular weight and shape. Reversibility of the dissociation was studied by examining the effect of neutralization on the sedimentation coefficient and by analysis of the ultracentrifuge 'schlieren' boundary curves, by a method discussed later.

* In all subsequent expressions of protein concentration the symbol % alone will denote w/v unless otherwise stated.

EXPERIMENTAL

Material

Carboxyhaemoglobin was prepared from freshly drawn or 1-week-old blood from a blood-bank, by the method of Jope & O'Brien (1949). Stock solutions contained the protein in distilled water and were stored at 0°.

Buffers

Reference to buffer solutions in the text will be made according to the first names appearing in the list below. The compositions, except for total molarities, were as given in the tables published by the authors listed and pH determinations were made with a glass electrode, against 0·05 M potassium hydrogen phthalate (pH 4·00) as standard.

Acetate: sodium acetate–HCl (0·1 M with respect to sodium acetate) (Vogel, 1951).

Acetate–urea: as above, containing 0·1 M urea.

Acetate–veronal: sodium acetate–sodium barbital–HCl (0·05 M with respect to each of the first two) (Michaelis, 1931).

Glycine–NaOH: glycine–NaCl–NaOH (0·1 M with respect to each of the first two) (Vogel, 1951).

Phosphate: Na_2HPO_4 anhydrous–KH_2PO_4 (0·033 M with respect to phosphate) (Vogel, 1951).

Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge at 900 rev./sec. using the bar-schlieren optical system (Baldwin, 1953). A tungsten-filament lamp was used as light source with a 600 mμ. filter. Sedimentation coefficients were calculated by the method of Cecil & Ogston (1948) and are expressed in the text as $10^{13} \times$ value in c.g.s. units reduced to 20° and solution in water. Samples in acetate–veronal and phosphate buffers were prepared by diluting stock haemoglobin solution (in distilled water) with buffer and dialysing at 0–5° against several changes of buffer; those in acetate, acetate–urea and glycine–NaOH buffers by diluting stock solution with buffer to the required final concentrations immediately before a run.

Diffusion. This was measured in the Gouy diffusiometer (Middlesex Hospital) described by Creeth (1952) or in the Oxford Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948). Diffusion coefficients are expressed as $10^7 \times$ value in c.g.s. units, reduced to 20° and solution in water. Solutions were dialysed for 72 hr. at 0–5° before being used for diffusion measurements.

RESULTS

Sedimentation and diffusion coefficients at pH 6–11. Fifteen determinations in acetate–veronal and glycine–NaOH buffers at pH values ranging from 6 to 11, with protein concentrations from 0·75 to

1·0%, gave a mean sedimentation coefficient of 4·24 ± 0·07 (standard deviation). This standard deviation is similar to that found by Cecil & Ogston (1948) to represent the normal experimental variation: hence there was no evidence of pH dependence within this range.

Seven determinations in phosphate buffer pH 8, with protein concentrations from 0·25 to 1·0%, gave a mean diffusion coefficient of 6·9 ± 0·1. No concentration dependence was shown.

These values agree with the value of 4·18 for the sedimentation and of 6·9 for the diffusion coefficient, reported by Kegeles & Gutter (1951) and by Lamm & Polson (1936) respectively.

Sedimentation coefficients at pH *below* 6. Curve *A*, Fig. 1, shows that in acetate buffer, with protein concentration of 0·71%, the sedimentation coefficient begins to fall at pH 5·5–6·0. The curve begins to flatten out at pH 3·5. At this pH the solution darkened rapidly during the experiment with the onset of denaturation (see below).

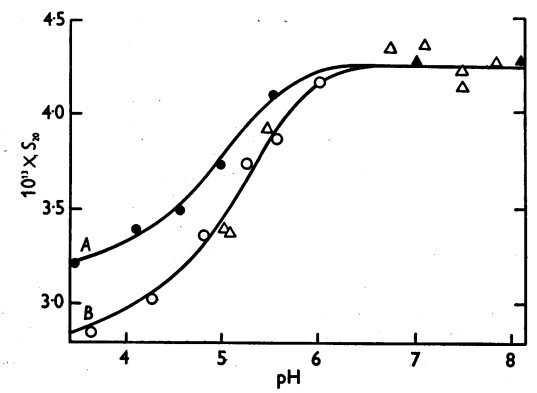

Fig. 1. Effect of pH below 6 on human carboxyhaemoglobin (0·71%). Curve *A*: acetate buffer alone; curve *B*: acetate buffer containing urea or barbiturate. Buffers indicated thus: ●, acetate; ○, acetate–urea; △, acetate–veronal; ▲, phosphate containing 0·1 M urea. Compositions as given in text.

Nature of buffer. Curve *B*, Fig. 1, shows that dissociation is facilitated by the addition of 0·1 M urea to the acetate buffers of curve *A*. In the presence of urea the curve is steeper and begins to flatten out at a lower value of sedimentation coefficient. The points obtained with acetate–veronal buffers, which at these pH values are saturated with respect to the barbituric acid, fit curve *B* remarkably well.

The relative effects of phosphate, acetate and acetate–urea buffers at pH 5·42 are compared in Table 1.

Effect of salt concentration. Table 2 shows the sedimentation coefficients obtained in acetate buffer, pH 4·80, in the presence of NaCl at concentrations 0 to 1·0 molar. In this range no significant effect on the sedimentation coefficient was observed.

Table 1. *Depression of sedimentation coefficient at* pH 5·42 *by different buffers*

Molarities as listed in text.

Buffer	$10^{13} \times S_{20}$
Phosphate	4·18
Acetate	4·03
Acetate–urea	3·81

Table 2. *Sedimentation coefficients at different salt concentrations*

Acetate buffer pH 4·80.

NaCl molarity	$10^{13} \times S_{20}$
0·0	3·65
0·2	3·74
0·5	3·62
1·0	3·69

Effect of protein concentration. The sedimentation coefficients in acetate buffer at pH 4·96 were found to be 3·73 with 0·71% protein and 3·01 with 0·21%; in acetate–urea buffer at pH 4·94 they were 3·67 with 1·74% protein and 3·40 with 0·35%. A less direct comparison could be made of the sedimentation coefficient of 2·99 obtained for 0·47% protein in acetate–veronal buffer at pH 4·58 with the corresponding value of 3·18 given by 0·71% protein on curve *B*, Fig. 1. Thus the degree of dissociation appears to increase with dilution.

Reversibility of dissociation. This was investigated by three methods.

(*a*) A sample of carboxyhaemoglobin was dialysed 24 hr. against acetate–veronal buffer, pH 5·0, the sedimentation coefficient determined, and the sample redialysed against this buffer at pH 7·5 for a further 48 hr. The sedimentation coefficient fell to 3·39 at pH 5·0 and returned to 4·23 at pH 7·5. In a second sample dialysed 48 hr. against acetate–urea buffer, pH 4·72, and then redialysed 72 hr. against phosphate buffer, pH 7·06, the sedimentation coefficients found were 2·70 and 4·18 respectively.

(*b*) A sample of carboxyhaemoglobin was prepared by diluting the stock solution with acetate–urea buffer of pH 3·60 immediately before a sedimentation run. At the end of the run it was neutralized by the addition of phosphate buffer and an amount of NaOH equivalent to that of HCl initially present. The sedimentation coefficient at pH 3·60 was 2·85 and at pH 7·50 rose to 3·87.

Thus, after exposure to pH 3·60 for 1 hr. at room temperature, the sedimentation coefficient did not completely return to normal.

(*c*) Boundary spread in the ultracentrifuge was analysed by the method discussed below. By the criteria of this method human carboxyhaemoglobin appeared to be homogeneous in acetate–veronal buffer, at pH 6·73, 7·1, 7·51, 7·84 and 8·98 as well as at pH 5·06. The significance of the last finding in relation to the reversibility of dissociation is discussed.

Effect of copper on dissociation. The addition of copper acetate to the acetate buffer, pH 4·94, with which carboxyhaemoglobin was diluted immediately before a sedimentation run, produced no significant effect on the sedimentation coefficient, with molar ratios of copper to haemoglobin (calculated for mol.wt. = 60 000), up to 1·0 at a protein concentration of 0·425 % (see Table 3), or in the presence of urea, with copper:haemoglobin ratios up to 34·0 at a protein concentration of 0·35 % (see Table 5).

Table 3. *Effect of copper on dissociation of native haemoglobin*

Protein 0·425 %. Acetate buffer pH 4·94.

Copper/haemoglobin molar ratio	$10^{13} \times S_{20}$
0·0	3·09
0·1	3·06
0·33	3·05
1·0	3·09

Table 4. *Effect of copper on dissociation of partially denatured haemoglobin*

Protein concentration 0·425 %. Acetate buffer pH 4·94.

Copper/haemoglobin molar ratio	Days stored at −20°	$10^{13} \times S_{20}$
0·0	30	3·02
0·74	0	3·22
1·0	30	3·30
2·0	33	3·19
3·0	34	3·21
4·0	34	3·15
0·0	34	3·02

However, it was found that with carboxyhaemoglobin which had been exposed to pH 4·94 at 0–5° for about a week the sedimentation coefficient had risen from 2·74 (Table 5, line 2) to 3·02, presumably due to the onset of denaturation. The addition of copper to this sample produced a further rise in the sedimentation coefficient, to a mean value of 3·21, which was already maximal at a copper:haemoglobin ratio of 0·74 and did not vary significantly at copper:haemoglobin ratios from 0·74 to 4·0. In order to eliminate the possibility of the protein denaturing further during the course of these experiments, the sample was divided into a number of portions which were stored in separate test-tubes at −20° until required. A sedimentation coefficient of 3·02 was again obtained in the absence of copper at the end of the series, thus indicating that no further denaturation had occurred during storage at −20° (see Table 4).

Maximum dissociation. Table 5 shows the sedimentation coefficients so far obtained under conditions of pH or protein concentration which would favour a high degree of dissociation. Both this Table and curve *B*, Fig. 1, suggest that about 2·7 may represent a limiting value for the sedimentation coefficient.

Diffusion coefficients. Two sets of determinations were carried out: the first on the effect of low pH on the diffusion coefficient with a protein concentration of 0·71 %, the second on the concentration dependence of dissociation at pH 4·88. The results are shown in Tables 6 and 7.

It will be seen that the diffusion coefficient rises with fall of pH in the range in which the reverse effect is shown by the sedimentation coefficient. There appears also to be a small rise in diffusion coefficient with dilution; in view of the reproducibility at the higher concentrations, within ± 0·03, this rise is probably significant.

DISCUSSION

These studies have established that the human carboxyhaemoglobin molecule dissociates at pH values below about 6·0, and that this dissociation

Table 5. *Sedimentation coefficients of highly dissociated carboxyhaemoglobin*

Protein concentration (g./100 ml.)	pH	Buffer	$10^{13} \times S_{20}$
0·71	3·60	Acetate–urea	2·85
0·425	4·94	Acetate	2·74
0·35	4·94	Acetate–urea dialysed 24 hr.	2·79
0·35	4·94	Acetate–urea with copper: Cu/Hb = 34·0	2·69
0·35	4·94	Acetate–urea with copper: Cu/Hb = 4·3	2·75
0·35	4·94	Acetate–urea with copper: Cu/Hb = 1·4	2·74
0·35	4·94	Acetate–urea with copper: Cu/Hb = 0·57	2·74
0·35	4·94	Acetate–urea with copper: Cu/Hb = 0·51	2·72
0·35	4·94	Acetate–urea	2·70

appears to be facilitated by urea and by barbiturate.

It has been claimed that urea affects the shapes of horse albumin and myoglobin molecules (Cohn & Edsall, 1943), but it is unlikely that the additional reduction in sedimentation coefficient of human carboxyhaemoglobin, at low pH, produced by urea is due to this effect, in view of the relatively large changes in axial ratio or hydration that would be required to explain the changes in sedimentation coefficient (e.g. 8% at pH 5) which occur in passing from curve *A* to curve *B*, Fig. 1.

Table 6. *Effect of low* pH *on the diffusion coefficient of carboxyhaemoglobin*

Protein concentration 0·71%.

Buffer	pH	$10^7 \times D_{20}$
Acetate–veronal	5·15	7·35
Acetate–veronal	5·15	7·35
Acetate–urea	4·95	7·58
Acetate–veronal	4·88	7·69
Acetate–veronal	4·88	7·74

Table 7. *Effect of haemoglobin concentration on diffusion coefficient*

Acetate–veronal buffer pH 4·88.

Protein concentration (g./100 ml.)	$10^7 \times D_{20}$
0·71	7·69
0·71	7·74
0·48	7·80
0·48	7·83
0·24	7·85
0·24	8·02

Moreover, normal values for sedimentation coefficient were obtained in phosphate buffers containing 0·1 M urea at pH 7·0 and 8·0 (see Fig. 1). It is interesting to note that the same reduction of sedimentation coefficient is produced by 0·1 M urea as by 0·05 M (effectively saturated) barbituric acid. The explanation may lie in the chemical relationship of these two compounds.

The reversibility of the dissociation was indicated by the return of the sedimentation coefficient to its normal value when the pH was raised from below 5·0 to above 7·0 by dialysis. However, there was evidence that below pH 4, in the presence of urea, irreversible denaturation sets in rapidly, for on neutralization after an hour's exposure to pH 3·60 the sedimentation coefficient did not entirely return to its normal value.

The fact that sedimentation coefficients had reached their new values within 20 min. of lowering the pH—by the method of diluting with buffer immediately before a sedimentation run—indicates that the dissociation is rapid. The rapidity with which equilibrium between the undissociated molecule and its dissociated products is attained is further demonstrated by analysis of the boundary schlieren curves, by the method of Baldwin (1953), based on that of Williams, Baldwin, Saunders & Squire (1952). This involves computation of the second moment of the distribution of material in the boundary. Correction is first applied for the effects of the non-uniform centrifugal field and for the dependence of the sedimentation rate on concentration, based on results of Kegeles & Gutter (1951) by the following relationship:

$$\sigma^2_{\text{corrected}} = \sigma^2 + 2\int_0^t \sigma(2\omega^2 x . \kappa\Delta c - \omega^2 S\sigma)\, dt,$$

where

σ^2 = apparent second moment, obtained by the height and area method.

ω = angular velocity of rotation.

S = sedimentation coefficient under the conditions of the experiment.

κ = slope of the sedimentation coefficient/concentration curve.

$\Delta c = 0{\cdot}34 \times$ concentration = difference between concentrations at the centre of the boundary and at the standard deviation of the concentration gradient curve.

x = distance of centre of boundary from centre of rotation.

To evaluate the integral, a quantity q defined by

$$q = \sigma\omega^2(2x\kappa\Delta c - S\sigma)$$

was calculated at the time of each photograph, and the integral replaced by

$$2\int_0^t q\, dt = 2\left[\frac{q_0+q_1}{2} + \frac{q_1+q_2}{2} + \ldots\right]\Delta t.$$

This corrected spreading of the boundary of a homogeneous solute should represent a pure diffusion process, i.e. should be linear with time. The effect of heterogeneity, uncomplicated by reversible dissociation, is to cause an additional variation of $\sigma^2_{\text{corr.}}$ linear with t^2: appreciable heterogeneity should therefore give a $\sigma^2_{\text{corr.}}/t$ plot showing an upward (positive) curvature. The results of this analysis (see Fig. 3 in the succeeding paper) show that $\sigma^2_{\text{corr.}}$ appears to be linear with t whether or not dissociation is taking place. This appearance of homogeneity could only result if equilibrium between the two forms were established rapidly in relation to their differential rates of migration. In the absence of dissociation, the slope of $\sigma^2_{\text{corr.}}$ against t should measure $2D$; D is the diffusion coefficient. Values of D obtained in this way at pH values above 6 lay between 7·5 and 8·0. Their average

value was 10% higher than the value directly determined (6·9 ± 0·1). This difference is probably due to systematic errors in measuring the optical record. At pH 5·06 the slope of $\sigma^2_{corr.}$ against t measured the spreading due to the combined effects of diffusion and heterogeneity: this could be expressed as an apparent diffusion coefficient D', of value 13·7.

Copper appears to induce a re-association of dissociated haemoglobin which has been partially denatured. The fact that native haemoglobin is not so affected suggests that groups, capable of combining with copper, are released during the process of denaturation. Recent work by Ingram (1955) showed that denaturation of human haemoglobin by sodium dodecyl sulphate in a molar ratio of 200:1 is accompanied by an increase of free SH groups, detectable by silver nitrate, from 4 to 8. This suggests that SH groups may be implicated in this copper effect.

The molecular weight of human haemoglobin, computed from the sedimentation and diffusion results, using the equation of Svedberg & Pedersen (1940) and assuming a partial specific volume of 0·749 was 59400 with a frictional ratio f/f_0 of 1·18. Wyman (1948) quotes a value 63000 for the molecular weight. The difference of this from the present value depends almost entirely on the high value used by Wyman for the sedimentation coefficient (4·48, Svedberg & Pedersen, 1940); this value is certainly too high (see Cecil & Ogston, 1948; Kegeles & Gutter, 1951). The average molecular weights and frictional ratios at the lower pH values were similarly calculated by combining diffusion results with corresponding sedimentation values obtained by interpolation in curve B (Fig. 1) for haemoglobin concentrations of 0·71%, or at lower concentrations by combining sedimentation results for 0·22% in acetate–urea with diffusion results for 0·24% in acetate–veronal buffer. The results are given in Table 8. Dissociation appears to be accompanied by a small, possibly insignificant, increase in frictional ratio. Table 8 also gives the degrees of dissociation at different pH values, calculated from the equation: $\alpha = (S_A - \bar{S})/(S_A - S_B)$, on the assumption that the molecule dissociates into identical halves of sedimentation coefficient 2·6. (For the derivation of this equation see succeeding paper.) This theoretical value for the sedimentation coefficient of a half-molecule of slightly greater frictional ratio than the whole agrees well with the lowest value of 2·69 observed experimentally. Using the computed values for the degrees of dissociation and the value of 8·45 for the diffusion coefficient of the half-molecule obtained from the relationship $D_B = 2D_A S_B/S_A$ (where suffixes A and B denote the whole or the half-molecule respectively) the average diffusion coefficient, $\bar{D}$, corresponding to any given degree of dissociation was calculated from the equation (Ogston, 1949):

$$1/\sqrt{\bar{D}} = \alpha/\sqrt{D_B} + (1-\alpha)/\sqrt{D_A}$$

(which applies to diffusion coefficients determined by the Gouy method). The results, shown in Table 8, agree very well with those determined experimentally.

Table 8. *Summary of principal results: sedimentation coefficients, diffusion coefficients, molecular weights and frictional ratios of carboxyhaemoglobin for different degrees of dissociation*

Buffer concentrations as listed in the text.

Protein concentration (g./100 ml.)	Buffer	pH	$10^7 \times D_{20}$ found	$10^7 \times D_{20}$ calculated	$10^{13} \times S_{20}$	Average molecular weight	Degree of dissociation	f/f_0
0·71	Phosphate	8·0	6·91	—	4·24	59 400	0	1·18
0·71	Acetate–veronal	5·15	7·35	7·48	3·56*	46 800	0·41	1·19
0·71	Acetate–urea	4·95	7·58	7·59	3·41*	43 600	0·51	1·19
0·71	Acetate–veronal	4·88	7·72	7·68	3·36*	42 100	0·54	1·19
0·24	Acetate–veronal	4·88	7·94	—	—	—	—	—
0·22	Acetate–urea	4·88	—	8·03	3·00	36 600	0·76	1·20

* From curve *B*, Fig. 1.

SUMMARY

1. Measurements have been made of the sedimentation and diffusion of human carboxyhaemoglobin, over ranges of pH, buffer composition, concentration and in the presence and absence of urea or copper.

2. The results show that these properties of the protein are independent of conditions between pH 6 and 11; between pH 3·5 and 6 a rapidly reversible dissociation occurs into particles of about half the particle weight and about the same frictional ratio as that of the undissociated material. Below pH 3·5, or on prolonged exposure to pH 5, an irreversible denaturation occurs.

3. The dissociation is increased by dilution, by lowered pH and by the presence of urea and barbitone.

4. Copper induces reassociation of partially denatured material but is without effect on native haemoglobin.

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The sedimentation measurements were made in the Department of Biochemistry, University of Oxford.

REFERENCES

Baldwin, R. L. (1953). Thesis, University of Oxford.
Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides as Ions*, p. 527. Amer. Chem. Soc. Monogr. Ser. N.Y.
Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. Roy. Soc.* A, **192**, 382.
Creeth, J. M. (1952). *Biochem. J.* **51**, 10.
Ingram, V. M. (1955). *Biochem. J.* **59**, 653.
Jope, H. M. & O'Brien, J. R. P. (1949). *Haemoglobin*, p. 269. Papers presented at the Sir Joseph Barcroft Memorial conference Cambridge, 1948.
Kegeles, G. & Gutter, F. J. (1951). *J. Amer. Chem. Soc.* **73**, 3770.
Lamm, O. & Polson, A. (1936). *Biochem. J.* **30**, 528.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Moore, D. H. & Reiner, L. (1944). *J. biol. Chem.* **156**, 411.
Ogston, A. G. (1949). *Proc. Roy. Soc.* A, **196**, 272.
Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*. Oxford: The Clarendon Press.
Vogel, A. I. (1951). *A Textbook of Quantitative Inorganic Analysis*, p. 869. London: Longmans Green and Co.
Williams, J. W., Baldwin, R. L., Saunders, W. M. & Squire, P. G. (1952). *J. Amer. chem. Soc.* **74**, 1542.
Wyman, J. (1948). *Advanc. Protein Chem.* **4**, 454.

Boundary Spreading in the Migration of a Solute in Rapid Dissociation Equilibrium. Theory and its Application to the Case of Human Haemoglobin

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It has been generally realized that no resolution of boundaries will occur in moving boundary transport experiments (sedimentation or electrophoresis) where two or more components are in an association–dissociation equilibrium which is established rapidly compared with the differential rate of migration. A single boundary will be observed which moves with the weight–mean velocity of its components (Tiselius, 1930), and which spreads more rapidly than is accounted for by diffusion. Qualitative discussions of this phenomenon have been given by Longsworth & MacInnes (1942), Alexander & Johnson (1949), Gilbert (1953) and Ogston (1953). Ogston (1946) attempted a quantitative treatment for the case of electrophoretic migration, but his allocation of a constant 'mean life' to each component is an over-simplification. Typical behaviour of this kind has been observed with a number of protein systems; Klotz (1953) mentions most of the earlier examples. Recent observations are those of Smith, Kimmel & Brown (1954) on papain and Field & O'Brien (1955) on human haemoglobin.

In this paper a theoretical treatment is given and applied to human haemoglobin.

THEORY

Qualitative. It is easy to see, in a qualitative way, that a mobile equilibrium between two or more forms, which migrate at different rates, will lead to a boundary spreading in addition to that due to diffusion. At any moment the solute will be distributed amongst its various forms. Provided that these forms have finite mean lives, a differential migration will occur during the next short interval of time. If, after this interval, there were a necessary reversion of fast into slow, and vice versa, the resolution produced in the first interval would be exactly reversed in the next interval. However, the statistical view of the nature of such a dynamic equilibrium demands that the behaviour of particles should be independent of their immediately previous history. The spreading produced during the first interval will therefore remain, and will be increased by similar spreading in the next interval, and so on. Evidently the spreading will be greater, as the difference of velocities between the forms and their mean lives are greater.

Quantitative. For simplicity it is assumed that the solute can exist in two states, A and B, in which it migrates at different velocities V_A and V_B, and that these states are interconvertible

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

at a rate sufficient for equilibrium between them to be maintained in spite of their differential migration. Thus at all points

$$k_1[A] = k_2[B],$$