We wish to thank Dr P. A. Charlwood and Mr J. R. Hall for their assistance with the Middlesex Hospital and Oxford Gouy diffusiometers, and Mr G. E. Newman for technical assistance in part of this work.

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

By E. O. FIELD and A. G. OGSTON
Department of Biochemistry (Radcliffe Infirmary), and Department of Biochemistry, University of Oxford

(*Received* 22 *November* 1954)

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

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

or, if the fraction of the total concentration c in the form B is α

$$k_1(1-\alpha)=k_2\alpha. \quad (1)$$

Baldwin (1953*a*) has shown for sedimentation that the rate of migration of the median of a boundary formed by polydisperse material is equal to the weight-mean rate of migration in the region of uniform composition ahead of the boundary; the same conclusion should apply to the descending boundary in electrophoresis. Thus, if α_0 is the fraction in form B and V_{0A}, V_{0B} are the velocities of A and B in the region of uniform composition, the median of the boundary moves with velocity

$$\bar{V}=(1-\alpha_0)\,V_{0A}+\alpha_0 V_{0B} \quad (2)$$

(cf. Tiselius, 1930). This median velocity provides a frame of reference with respect to which the relative movements of A and B may be measured. At any point in the boundary

$$\left.\begin{aligned} v_A&=V_A-\bar{V},\\ v_B&=V_B-\bar{V}.\end{aligned}\right\} \quad (3)$$

v_A, v_B and V_A, V_B refer to this particular point in the boundary; in general, they are not constant quantities, but are functions of concentration and of other quantities which may vary through the boundary.

We may now specify the flux of material across some arbitrary plane within the boundary, defined by position x measured relative to the moving frame of reference, moving at velocity $\bar{V}$. Suppose that $V_A>V_B$, so that v_A is positive and v_B negative. Only material in form A can cross the plane in a positive direction, and in form B in a negative direction. The rate of positive transport across the plane is then $v_A[A]$ and that of negative transport $v_B[B]$. If the equilibrium is established infinitely rapidly, the values of $v_A[A]$ and $v_B[B]$ are those at the plane; but if equilibrium is not established infinitely rapidly, the values of v_A and [A] refer, not to the plane, but to a mean position $-\delta_A/2$ on the negative side of the plane where δ_A is the mean distance moved by A during its mean life in this form. (If equilibrium is infinitely rapidly established, the mean life becomes infinitely small.) Now the mean life of A,

$$\bar{\tau}_A=1/k_1,$$

so that

$$\delta_A=v_A\bar{\tau}_A=v_A/k_1. \quad (4a)$$

Similarly, v_B and [B] refer to a position $\delta_B/2$ on the positive side of the plane and, using Eqn. 1

$$\delta_B=-v_B\bar{\tau}_B=-v_B/k_2=-v_B\alpha/k_1(1-\alpha). \quad (4b)$$

If subscript x indicates the value at the reference plane, the positive flux (J_+) is

$$\begin{aligned} J_+&=v_{xA}[A]_x-\frac{\delta_A}{2}\frac{d}{dx}(v_A[A])\\ &=v_{xA}[A]_x-\frac{v_{xA}}{2k_1}\frac{d}{dx}(v_A[A]); \end{aligned}$$

similarly, the negative flux (J_-) is

$$\begin{aligned} J_-&=v_{xB}[B]_x+\frac{\delta_B}{2}\frac{d}{dx}(v_B[B])\\ &=v_{xB}[B]_x-\frac{v_{xB}\alpha_x}{2k_1(1-\alpha_x)}\frac{d}{dx}(v_B[B]). \end{aligned}$$

Then the total flux across the plane, due to the differential migration is

$$\begin{aligned} J_T&=J_++J_-\\ &=\{v_A[A]+v_B[B]\}_x-\frac{1}{2k_1}\left\{v_A\frac{d}{dx}(v_A[A])\right.\\ &\qquad\left.+\frac{v_B\alpha}{(1-\alpha)}\frac{d}{dx}(v_B[B])\right\}_x. \end{aligned} \quad (5)$$

Eqn. 5 shows the salient characteristics of this type of transport; namely, that it is the sum of two terms, one containing only the velocities and concentrations, the other containing their differential coefficients. To evaluate it requires knowledge of the values of these quantities at any chosen point within the boundary.

In order to make Eqn. 5 more useful, we now make two specific assumptions: (i) that V_A, V_B are constant and equal to V_{0A} and V_{0B}; in most cases these quantities will, at the worst, vary much less rapidly through the boundary than will the concentrations; (ii) that A dissociates into n equal fragments when it forms B; the equilibrium may then be expressed

$$K=\frac{(c\alpha)^n}{c(1-\alpha)}. \quad (6)$$

From this it follows that

$$d\{c(1-\alpha)\}=\frac{n(1-\alpha)}{\alpha}\,d\{c\alpha\}. \quad (7)$$

Replacing v_A and v_B in Eqn. 5, using Eqns. 2, 3 and 7, and replacing [A] and [B] by $c(1-\alpha)$ and $c\alpha$, we obtain

$$\begin{aligned} J_T&=(V_{0A}-V_{0B})\,(\alpha_0-\alpha)\,c\\ &\quad-\frac{(V_{0A}-V_{0B})^2}{2k_1}\left\{\frac{n\alpha_0^2(1-\alpha)^2+(1-\alpha_0)^2\alpha^2}{\alpha(1-\alpha)}\right\}\frac{d(c\alpha)}{dc}\frac{dc}{dx}, \end{aligned} \quad (8a)$$

which may be represented by

$$J_T=\xi c-\zeta\,dc/dx, \quad (8b)$$

ξ and ζ representing the complex coefficients in Eqn. 8*a*.

Eqn. 8 again shows that the flux across a plane within the boundary is the sum of two terms. Both, when finite, are negative in sign. The values of both terms are zero except within the boundary, and they therefore express redistribution of material within the boundary, not a flux through the boundary as a whole. The first term is proportional to concentration, so that its value is

independent of time; the second term is proportional to dc/dx (as is flux due to diffusion) and its value therefore decreases with time as the boundary spreads.

It is interesting to note that if $n=1$, then α is constant $=\alpha_0$; the mean lives of A and B are then independent of concentration and the flux becomes

$$J_T = -\frac{(V_{0A}-V_{0B})^2}{k_1}\alpha(1-\alpha)\,dc/dx,$$

which is identical with the corresponding flux obtained from the equation given by Ogston (1946).

In order to give a complete description of boundary spreading it is necessary also to allow for the flux due to diffusion. It is assumed that this flux can be added to that given by Eqn. 8, that is

$$\begin{aligned} J &= J_T + J_D \\ &= \xi c - \zeta dc/dx - \bar{D}\,dc/dx, \end{aligned} \qquad (9)$$

$\bar{D}$ being the weight-mean diffusion coefficient, where $\bar{D}=(1-\alpha)D_A+\alpha D_B$.

Computation of boundary spreading

Although Eqn. 9 cannot be directly integrated, all the necessary values are available from the results obtained on human haemoglobin, in the preceding paper, to enable an approximate integration to be performed. The case considered corresponds with the experiment on haemoglobin at pH 5·06 in acetate–veronal buffer. The computation was performed in the following stages: (i) using the mean sedimentation coefficient 3·4 together with the sedimentation coefficients of the undissociated and fully dissociated forms, 4·3 and 2·7 respectively, in Eqn. 2 gives $\alpha_0=0{\cdot}56$. Putting this value into Eqn. 6 with $n=2$ and $c=1{\cdot}0$ gives $K=0{\cdot}71$. From K, a curve of α against c was calculated. (ii) Using the α–c relationship values of ξ and $(\zeta+\bar{D})$ (Eqn. 9) were calculated and plotted against c (Fig. 1). In order to calculate ζ (Eqn. 8*b*), some assumption had to be made about the value of k_1; the only evidence on this (preceding paper) is that it is greater than 1/20 min.$^{-1}$; a value of 1/200 sec.$^{-1}$ was assumed. V_{0A}, V_{0B} were assumed to be given by $\omega^2 rS$, where ω (angular velocity of rotation) was taken as $5{\cdot}7\times10^3$ radians sec.$^{-1}$; r (radius of rotation at the boundary) was taken as 5·5 cm.; S (sedimentation coefficient) was taken as 4·3 and $2{\cdot}7\times10^{-13}$, respectively. Finally, taking D_A as the measured value at pH 8·0 of 6·9 cm.^{2} sec.$^{-1}$, D_B as $2D_AS_B/S_A$ (this follows from the assumption that $n=2$), $\bar{D}$ was calculated as a function of c. (iii) The boundary was assumed at 4·5 sec. to have the simplified form shown in Fig. 2; the unit values of δx were taken as 5×10^{-3} cm. (iv) The amount of material transported across the planes -1, 0, $+1$ in the course of the ensuing 7·5 sec. were then calculated using Eqn. 9. (v) The resulting changed average concentrations in the four laminae were thus obtained, and the new form of the boundary was plotted. (vi) A further pair of planes $(-2, +2)$ were added. Stages (iv), (v) and (vi) were then reiterated, with the time intervals chosen so as to give equal increments of $t^{\frac{1}{2}}$, corresponding approximately with a total spreading

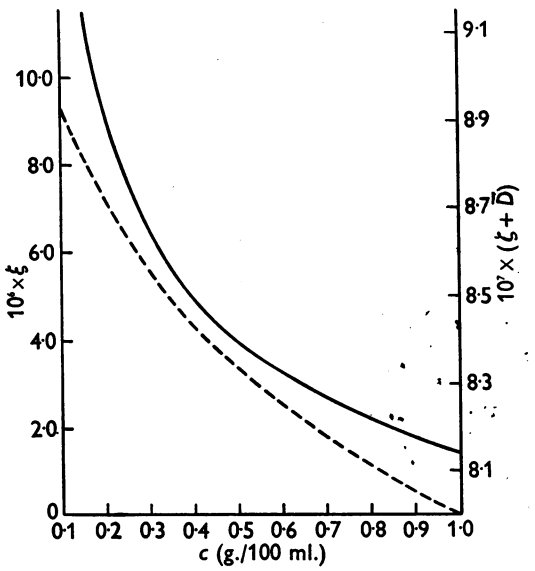

Fig. 1. Curves of ξ (dashed line) and of $(\zeta+\bar{D})$ (full line) plotted against c, g./100 ml. (Eqn. 9).

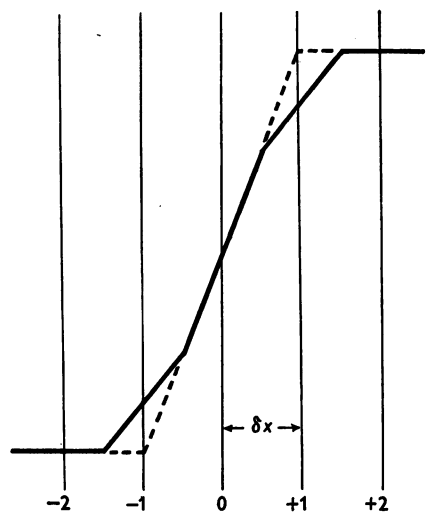

Fig. 2. Method of integrating Eqn. 9: full line, assumed initial form of boundary at 4·5 sec. Ordinates, concentration; abscissa, distance x. Constructional lines dashed. Vertical lines represent planes enclosing laminae of thickness $\delta x=5\times10^{-3}$ cm.

coefficient of $12{\cdot}3 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, taken from the experimentally determined value of $13{\cdot}7 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, corrected for the systematic error in measurement. As soon as the number of laminae justified it, smoothed curves were drawn through the calculated values of c and dc/dx, and were used for the ensuing stage. The computation was carried through nineteen stages, the total elapsed time being 4500 sec.

Shape of boundary schlieren curves

In view of the small concentration-dependence of sedimentation rate, the boundary curves of homogeneous haemoglobin (pH > 6) should be symmetrical; in fact they were found to be slightly asymmetrical, the trailing limb being steeper than the advancing limb. The ratio of slopes at the points of inflexion was independent of time; the effect was, therefore, almost certainly the result of the optical system being slightly out of focus. The experimental boundary curves obtained at pH 5·06 showed a changing ratio of slopes, the trailing limb starting steeper and becoming less steep than the advancing limb. Correction for the optical distortion (on the basis of that observed at pH > 6) gave curves initially symmetrical, and becoming increasingly asymmetrical with time, the trailing limb being the less steep.

DISCUSSION

Rate of boundary spreading. Williams, Baldwin, Saunders & Squire (1952) and Baldwin (1953*b*) have shown that a plot of σ^2 against t, after correction of σ^2 for the effects of variation of the centrifugal field and of variation of sedimentation rate with concentration, will be linear for a homogeneous substance and will show a curvature (its slope increasing with t) if two or more components are present which sediment at different rates. Linearity of this plot may therefore provide evidence of homogeneity with regard to rate of sedimentation. If, in the present case, there were not a mobile equilibrium between the two forms of haemoglobin (that is, $k = 0$), then for the case where $\alpha = 0{\cdot}56$, σ^2 is given by

$$\sigma^2 = 2(0{\cdot}56D_B + 0{\cdot}44D_A)\,t + 0{\cdot}56 \times 0{\cdot}44\{r\omega^2(S_A - S_B)\}^2 t^2.$$

This has a very obvious curvature (Fig. 3), its slope at $t = 0$ being 15·6 and at $t = 4500$ sec. $34{\cdot}0 \times 10^{-7}$. If there were an infinitely rapid equilibrium between the two forms ($k = \infty$), the system would behave as a single homogeneous component.

Evidently as k decreases from ∞ to 0, an increasing curvature of the σ^2, t plot is to be expected; however, it is not easy to see from Eqns. 8 and 9 at what value of k curvature will become appreciable. In the case of $k = 1/200$ sec.$^{-1}$, which does not represent a very high rate of association–dissociation, our computations show (Fig. 3) no obvious curvature up to 4500 sec. One must conclude that this type of analysis will therefore not necessarily reveal heterogeneity if there is a moderately rapid equilibrium between the different forms of the solute.

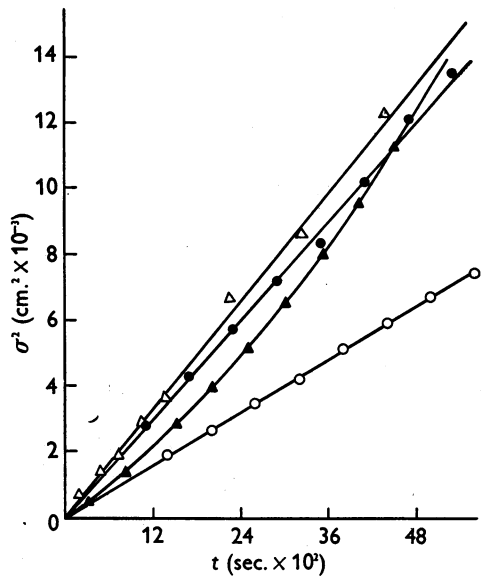

Fig. 3. Plots of σ^2 against t. ○, pH 7·5 observed; ●, pH 5·06 observed; △, pH 5·06 predicted for a system in mobile equilibrium where $k = 5 \times 10^{-3}$; ▲, pH 5·06 predicted for a system where $k = 0$ and $\alpha = 0{\cdot}56$.

The computed value of the spreading coefficient ($d\sigma^2/2dt$) of $13{\cdot}5 \times 10^{-7}$ is to be compared with the corrected observed value of $12{\cdot}3 \times 10^{-7}$ (see above). The agreement is satisfactory in view of the uncertainty of the value of k. Diffusional spreading alone would account for about 8×10^{-7}, which shows that in this case the spreading expressed by Eqn. 8 accounts for about 40 % of the whole. This proportion would increase with decrease of k.

Shape of the boundary. The form of Eqn. 8 suggests a skewing of the boundary in the sense of the trailing limb being the less steep, as is observed. The quantity $\mu_3/\mu_2^{3/2}$ (Aitken, 1939) which is a measure of skew is 0·23 for the observed boundary at 4800 sec. and 0·15 for the computed boundary at 4500 sec.

SUMMARY

1. A theoretical treatment of boundary spreading in a rapidly dissociating system is given, which is valid for sedimentation or electrophoresis. The

case considered is $A \rightleftarrows nB$, but the treatment could be easily extended. It leads to a differential equation for transport of material across a plane, due to the combined effects of differential migration velocities and diffusion.

2. Although the differential equation cannot be directly integrated, numerical integration can be performed. This has been done, using the various quantities appropriate to human haemoglobin at pH 5·06, available from the experimental results of the preceding paper; only the velocity constant of the dissociation has to be guessed.

3. The agreement between the calculated and observed spreading of the boundary is satisfactory. In particular it is shown that the shape of the boundary and the variations of its rate of spreading with time are remarkably insensitive to the heterogeneity caused by a fairly rapidly established dissociation equilibrium.

REFERENCES

Aitken, A. C. (1939). *Statistical Mathematics*, p. 37. Edinburgh: Oliver and Boyd.
Alexander, A. E. & Johnson, P. (1949). *Colloid Science*, p. 279. Oxford University Press.
Baldwin, R. L. (1953*a*). *Biochem. J.* **55**, 644.
Baldwin, R. L. (1953*b*). Thesis, University of Oxford.
Field, E. O. & O'Brien, J. R. P. (1955). *Biochem. J.* **60**, 656.
Gilbert, G. A. (1953). *Disc. Faraday Soc.* no. **13**, p. 159.
Klotz, I. M. (1953). In Neurath, H. & Bailey, K., *The Proteins*, p. 727. New York: Academic Press.
Longsworth, L. G. & MacInnes, D. A. (1942). *J. gen. Physiol.* **25**, 507.
Ogston, A. G. (1946). *Nature, Lond.*, **157**, 193.
Ogston, A. G. (1953). *Disc. Faraday Soc.* no. **13**, p. 165.
Smith, E. L., Kimmel, J. R. & Brown, D. M. (1954). *J. biol. Chem.* **207**, 533.
Tiselius, A. (1930). *Nova Acta Soc. Sci. upsal.* IV, **7**, no. 4, p. 1.
Williams, J. W., Baldwin, R. L., Saunders, W. M. & Squire, P. G. (1952). *J. Amer. chem. Soc.* **74**, 1542.

Clearing Factor and Lipase

By G. A. OVERBEEK and J. VAN DER VIES
Department of Pharmacological Research, N.V. Organon, Oss, The Netherlands

(*Received* 6 *December* 1954)

Since the work of Hahn (1943), it has been repeatedly shown that alimentary lipaemia disappears after the intravenous injection of heparin. It is also a well-established fact that this is not a direct effect of heparin, but that the administration of heparin gives rise to the appearance in the blood of another substance, which has been called 'clearing factor' (Anderson & Fawcett, 1950). Since then the work of other groups has brought to light many chemical and biological features of this interesting substance. One of the unsettled problems is the relationship between clearing factor and lipase. Clearing factor clears lipaemia *in vitro*, which heparin is unable to do, and during the clearing process fatty acids are liberated. This suggested that clearing factor might be a kind of lipase or an activator of this enzyme.

However, we and several other groups of investigators have failed to show an increase of the lipase activity in the plasma of rats and dogs after heparin administration, although there are investigators who hold the opinion that the lipase activity is enhanced. A cause of these divergent results might be that several types of fat-splitting enzymes (lipases) occur in the plasma of various species of animals, and that the activity of different enzymes may be measured depending on the substrate used and other conditions of the experiment.

Mendel, Myers, Uyldert, Ruys & Bruyn (1953) introduced the term 'ali-esterases' for enzymes which hydrolyse only esters of lower fatty acids (like tributyrin) but not esters of palmitic, oleic and other higher fatty acids. These ali-esterases are inhibited by sodium *p*-aminophenylarsonate (atoxyl), tri-*o*-cresyl phosphate (TOCP) and taurocholate. 'True' lipase degrades both esters of lower and higher fatty acids, is inhibited by quinine but not by atoxyl and TOCP, and is even activated by taurocholate. In this paper we employ the terms 'ali-esterase' and 'lipase' for these two separate enzymes and 'tributyrinases', when both are discussed. In human beings both types of tributyrinases occur in the plasma. Ali-esterases are produced in the liver, lipase in the pancreas (Srinivasan & Patwardhan, 1952). It will be shown that in other species of animals either one or other type occurs almost exclusively in the plasma.

The aim of this investigation was to ascertain whether ali-esterases or lipase are essential for the formation and/or action of clearing factor. For this purpose we studied first the influence of well-known tributyrinase inhibitors on the clearing process *in vitro*. Later rats were freed from ali-esterase activity by pre-treatment with TOCP, whereas in dogs the lipase activity of the plasma was reduced by pancreatectomy. Heparin was then injected