led Fruton (1948) to synthesize the diketopiperazine from D-leucine and L-tryptophan, but no antibacterial activity was encountered.

These findings indicate that growth inhibitory properties cannot be correlated with the mere presence of D-amino-acids in peptide linkage. At the outset of the present investigation it was considered likely that, as an additional requirement, the D-amino-acid should be flanked by a suitable pair of amino-acids; it was supposed that when this requirement was met optically selective antibiotic action might appear. A tripeptide possessing the D-amino-acid present in gramicidin S, flanked by the same two amino-acids as in the natural antibiotic peptide has, however, failed to show selective toxicity.

All the available evidence now supports the view expressed by Work (1948) that peptide antibiotics containing D-amino-acids are active, not because they have this character in common, but rather by virtue of the individual structure of the peptide.

In order to eliminate as far as possible the complicating factors of molecular size, and number and type of polar groups per molecule, it seemed desirable

to extend the present investigation by the syntheses of appropriate pentapeptides containing the complete amino-acid sequence of gramicidin S; the results of this investigation will be reported in a future communication.

SUMMARY

1. Dipeptides and tripeptides containing D-phenylalanine have been synthesized and compared as growth inhibitors with optically isomeric peptides containing L-phenylalanine.

2. The amino-acids in these peptides were arranged in the same sequence as in gramicidin S.

3. Limited antibiotic activity was observed with synthetic peptides, but no difference in activity was found between a tripeptide containing D-phenylalanine and one containing L-phenylalanine.

4. The significance of these results in relation to the origin of antibiotic activity in peptides is discussed.

We are indebted to Dr A. T. Fuller for the biological results summarized in Table 1.

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The Steady State, a Test for Electrophoretic Homogeneity

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The distribution of the ion concentrations across the boundaries formed by a solution of a homogeneous protein changes during electrophoresis. The tendency is for the boundary in one limb of the U-tube to become narrower and for that in the other to become broader, mainly on account of the differences in conductivity on both sides of each boundary (Longsworth & MacInnes, 1940), but also because the

mobility changes with the ionic strength. These effects may be enhanced or counteracted by changes in pH (Longsworth, 1947). If the ascending boundary of a leading ion has the tendency to become narrower, and this is generally the case with proteins at a pH removed from the isoelectric point, its behaviour during migration may be followed for an unlimited period of time, provided a countercurrent of buffer

solution of constant composition can be kept up through the U-tube. The factors responsible for the spreading of initially sharp boundaries have been discussed by Ogston (1946). Spreading can be due to thermal diffusion or to electrical heterogeneity. Considering a complex electrolyte, such as a protein, which is present in a number of ionic forms of different mobility, Ogston (1946) could show that the spreading of the boundary is formally equivalent to diffusion, except that it increases as the square of the potential gradient. Since, for a given potential gradient, the rate at which the boundary becomes broader decreases with increasing time while the tendency to sharpen remains constant being dependent only on the difference between the migration velocities of the protein ions at either side of the boundary, a steady state at which spreading and sharpening balance should be approached. The mathematical solution for the steady state occurring in the differential moving-boundary method for transport numbers of strong electrolytes has been given by Longworth (1943). In the following the theoretical shape of the boundary and the possibility of experimentally reaching and maintaining such a steady state with a protein will be examined.

THEORETICAL

Notation. V_0 = migration velocity of the protein ions at infinitely low concentration in the buffer solution; V = actual migration velocity of the protein ions; V_s = migration velocity of the boundary in the steady state; C = concentration of protein in g./100 ml.; C_0 = concentration of protein in the adjusted solution below the boundary; K , K_1 , K_2 , etc. = coefficients; x = vertical position in the U-tube; t = time; D' = apparent diffusion constant.

Derivation. Equation (1), which had been proposed as a first approximation valid for low colloid concentrations (Hoch, 1948), can be extended as in (2):

$$V = V_0(1 + KC), \quad (1)$$

$$V = V_0(1 + KC + KK_1C^2 + KK_1K_2C^3 + \dots). \quad (2)$$

This series converges if KC , K_1C , K_2C , etc. < 1 . For the particular case of serum albumin in phosphate buffer of pH 8, $I = 0.1$, it has been shown experimentally that K is about 0.06 at a protein concentration of 1 g./100 ml. and theoretically, on the basis of Svensson's (1946) boundary equation, that the variation in K from $C = 0$ to $C = 1$ g./100 ml. is 11 % or less (Hoch, 1948; Hoch-Ligeti & Hoch, 1948). This is equivalent to $K_1 = 0.11$ (or less) if the members of the series in (2) containing K_2 , K_3 , etc., are neglected. Equation (2) then becomes

$$V = V_0(1 + KC + KK_1C^2) = V_0(1 + KC(1 + K_1C)). \quad (3)$$

The following derivation of the shape of the curve of dc/dx against x will be based on equation (3) or on equation (1). The results should therefore be considered as approximations valid for K_2C , K_3C , etc. $\ll 1$.

If a boundary of a homogeneous protein moves in the steady state its velocity of migration is $V_s = V_0(1 + KC_0 + KK_1C_0^2)$. The amount of protein, per unit area of cross-section trans-

ported with the velocity $V = V_0(1 + KC + KK_1C^2)$ across a plane in the boundary in excess of that amount transported across a neighbouring plane with the velocity

$$V = V_0(1 + K(C + dC) + KK_1(C + dC)^2)$$

$$\text{is} \quad V_0(KdC + 2KK_1CdC) C dt.$$

The change in concentration therefore in the lamina bounded by the planes, with reference to a co-ordinate system moving with the velocity

$$V = V_0(1 + KC + KK_1C^2)$$

$$\text{is} \quad (dC)_1 = V_0(K + 2KK_1C) C \frac{dC}{dx} dt.$$

In the co-ordinate system moving with the velocity V_s , any point on the curve of C against x , which moves with a velocity $C < C_0$, lags behind by the distance

$$[V_0K(C_0 - C) + V_0KK_1(C_0^2 - C^2)] dt$$

corresponding to a change in concentration

$$(dC)_2 = [V_0K(C_0 - C) + V_0KK_1(C_0^2 - C^2)] dt \frac{dC}{dx}.$$

$(dC)_1 - (dC)_2$ is the change in concentration at a point in the system moving with the velocity V_s . Hence the change in concentration per unit time, due to the sharpening effect

$$\begin{aligned} \frac{dC}{dt} &= V_0(K + 2KK_1C) C \frac{dC}{dx} \\ &\quad - [V_0K(C_0 - C) + V_0KK_1(C_0^2 - C^2)] \frac{dC}{dx} \\ &= -V_0K(C_0 + K_1C_0^2 - 2C - 3K_1C^2) \frac{dC}{dx}. \end{aligned}$$

At any point the change in concentration per unit time, due to spreading, is given by

$$\frac{dC}{dt} = -D' \frac{d^2C}{dx^2},$$

where D' covers the effects of thermal diffusion, the spreading through the presence of different interconvertible ionic forms, mechanical disturbance and the effects brought about by the process of compensation. It is assumed to be constant.

In the steady state, the spreading and sharpening effects balance each other

$$-D' \frac{d^2C}{dx^2} = -V_0K(C_0 + K_1C_0^2 - 2C - 3K_1C^2) \frac{dC}{dx}. \quad (4)$$

Integrating once

$$\frac{dC}{dx} = \frac{V_0K}{D'} [(C_0 + K_1C_0^2) C - C^2 - K_1C^3] + m. \quad (5)$$

Since $dC/dx = 0$ for $C = 0$ and $C = C_0$ it follows that $m = 0$. Rearranging,

$$\begin{aligned} \frac{dC}{C \left(C^2 + \frac{1}{K_1} C - \frac{1}{K_1} C_0 - C_0^2 \right)} &= - \frac{V_0KK_1}{D'} dx, \\ \frac{dC}{C_1C_2C} + \frac{dC}{C_1(C_1 - C_2)(C - C_1)} - \frac{dC}{C_2(C_1 - C_2)(C - C_2)} \\ &= - \frac{V_0KK_1}{D'} dx, \end{aligned}$$

where C_1 and C_2 are the roots of

$$C^2 + \frac{1}{K_1} C - \frac{1}{K_1} C_0 - C_0^2 = 0, \quad C_1 = 0, \quad C_2 = - \left(C_0 + \frac{1}{K_1} \right).$$

Integration gives

$$-\frac{1}{C_0 \left(C_0 + \frac{1}{K_1}\right)} \log C + \frac{1}{C_0 \left(2C_0 + \frac{1}{K_1}\right)} \log (C_0 - C) - \frac{1}{\left(C_0 + \frac{1}{K_1}\right) \left(2C_0 + \frac{1}{K_1}\right)} \log \left(C + C_0 + \frac{K_1}{1}\right) = -\frac{V_0 K K_1}{D'} (x + \text{const.}) \quad (6)$$

If $K_1 C_0$ can be neglected relative to unity, equation (5) becomes

$$\frac{d^2 C}{dx^2} = \frac{V_0 K}{D'} (C_0 - 2C) \frac{dC}{dx} \quad (7)$$

Integrated once

$$\frac{dC}{dx} = \frac{V_0 K}{D'} (C_0 C - C^2) + m, \quad (8)$$

Fig. 1. Theoretical shapes of boundary patterns. *a*, calculated from equations (5) and (6) with $C_0 = 0.85$ and $K_1 = 0.3$; the value for K_1 was arbitrarily chosen to represent an experimental possibility. *b*, calculated from equation (10). *c*, curves of *a* and *b* superimposed. *d*, calculated from equations (5) and (6) with $C_0 = 0.85$ and $K_1 = 1.0$. The units for both the abscissae and ordinates are arbitrary.

where $m = 0$ as in equation (5); rearranging equation (8) and integrating

$$\left. \begin{aligned} \frac{dC}{C_0 C} + \frac{dC}{C_0 (C_0 - C)} &= \frac{V_0 K}{D'} dx, \\ \log \frac{C}{C_0 - C} &= \frac{V_0 K C_0}{D'} (x + n). \end{aligned} \right\} \quad (9)$$

If $x = 0$ is chosen for $d^2 C/dx^2 = 0$ and $dC/dx \neq 0$, by inserting equation (8) in equation (7),

$$\frac{d^2 C}{dx^2} = \left(\frac{V_0 K}{D'}\right)^2 (C_0 - 2C) (C_0 - C) C = 0;$$

this gives the solutions $C_1 = 0$, $C_2 = C_0$, $C_3 = C_0/2$. The maximum of the curve of dC/dx is at $C_3 = C_0/2$. Inserting this value in (9) n becomes zero and

$$\frac{C}{C_0 - C} = \exp\left(\frac{V_0 K C_0}{D'} x\right) \quad \text{and} \quad C = C_0 \frac{\exp\left(\frac{V_0 K C_0}{D'} x\right)}{1 + \exp\left(\frac{V_0 K C_0}{D'} x\right)}.$$

Inserting this value for C in (8) we obtain

$$\frac{dC}{dx} = \frac{V_0 K C_0^2}{D'} \frac{\exp\left(\frac{V_0 K C_0}{D'} x\right)}{\left[1 + \exp\left(\frac{V_0 K C_0}{D'} x\right)\right]^2} \quad (10)$$

This equation is formally equivalent to Longworth's (1943) equation $\frac{dc}{dh} = -\frac{a_2 i(\Delta c)^2}{4FD} \bigg/ \cosh^2 \frac{a_2 i \Delta c}{2FD} h$.

With the protein boundaries considered here the buffer salt concentration is greater above the boundary than it is below. If such a boundary, formed between the adjusted solution and the buffer, is left to diffuse without migration, the diffusion of the buffer salt, being faster than that of the protein, will cause the formation of two gravitationally unstable regions; the salt concentration in the region above the protein boundary will be smaller than that of the bulk of the buffer solution above it, and the salt concentration in the region below the protein boundary will be greater than that of the adjusted solution underneath. One must assume that con-

vection will result, and that the composition of the solutions on either side of the protein boundary will remain unchanged in the regions where the density gradient due to the change in the protein concentration is sufficiently small. Consequently, the variation in salt concentration across the boundary will tend towards becoming linear. On migration the distributions of the gradients of the buffer ion and the protein concentrations will approach each other in shape. The protein concentration curve, which has been derived without considering the effects just described, will be distorted from a sigmoid towards a linear form. This should result in a decrease of the protein concentration gradients in the centre of the boundary and an increase of the gradients at the tails. It is clear, therefore, that the spreading of the boundary arising from the diffusion of the buffer ions cannot be expressed by an additive term in the apparent diffusion constant D' . The calculation of the gradient curve with the help of a fictitious diffusion constant, together with the qualitative consideration of the effects of the diffusion of the buffer salt ions, serves to indicate the direction in

which the experimental curves would be expected to deviate from the error curve.

Fig. 1 *a, b* shows the plots of equations (5) and (10). In order to compare the curves with respect to the variation in the width with the relative height, all curves were plotted on such scales as to make the heights and the areas under the peaks equal. As can be seen from equation (10) changes in C_0 , V_0 , D' and K affect only the width and the height but not the characteristic shape of the curve. Equation (5) gives a slightly asymmetrical curve which is less steep on the side towards the low protein concentration than on the other side. Both curves differ little from each other as seen in Fig. 1 *c*, where they are superimposed. Compared with a Gaussian error curve these curves are narrower at the top and wider at the bottom. Increase of K_1 leads to a greater asymmetry and a greater deviation from the error curve, but the effect is slight, as can be seen in Fig. 1 *d*, where $K_1 = 1.0$ has been used. From equation (10) it follows that the width of the peak is inversely proportional to the migration velocity, i.e. the potential gradient, if all other conditions remain the same.

EXPERIMENTAL

Materials. (a) Oxyhaemoglobin (HbO_2). Normal human red cells were washed 4 times with 0.85% (w/v) NaCl, and lysed with 2–3 times their volume of water. To the clear supernatant was added 0.1 vol. of 0.33M- Na_2HPO_4 , the precipitate which formed was centrifuged (by centrifuging (Hoch, 1949) the turbid fast component described by Stern, Reiner & Silber (1945) was removed) and the supernatant diluted with buffer solution to the required concentration of HbO_2 . (b) Crystallized bovine serum albumin prepared by Armour Laboratories, Chicago, Illinois. The author is indebted to Dr H. Gutfreund for a sample of this material. (c) Serum from a blood donor who had hypertension. The protein pattern was normal but for the albumin peak which remained single on prolonged electrophoresis.

Electrophoresis and diffusion measurements. The experiments were made in a Tiselius apparatus equipped with a cylindrical lens schlieren optical system (Thovet, 1914; Philpot, 1938; Svensson, 1939). A diagonal wire was used to produce the patterns for subsequent tracing. Some of the patterns were also recorded with a diagonal edge because of their superior appearance in print. The light passing through the U-tube was horizontal and parallel. The focusing, particularly that of the U-tube on the plate, was checked by observing the patterns of the sharp initial boundaries. They were symmetrical and their axes were vertical to the base line. An error curve fitted to a diffusion boundary pattern obtained under the same conditions also showed that the optical set up was satisfactory.

The experiments with HbO_2 were made in solutions of 0.01M- Na_2HPO_4 . The fact that the phosphate solution has a negligible buffering capacity has no bearing on the present experiments since any pH effects are taken care of in the determination of K made under the same conditions. The experiments with albumin were made in phosphate of pH 8, $I = 0.1$. The conditions for the experiments with HbO_2 were chosen so as to give pronounced boundary anomalies and a relatively high migration velocity of the HbO_2 .

All runs were carried on beyond the time during which the ascending boundary migrated the distance of one limb of the U-tube and a constant current of buffer solution was kept up in the direction opposite to that of the migration of the boundary. In order to prevent the Cl^- in the bottom of the electrode vessels from reaching the U-tube a layer of 5–10 times concentrated phosphate solution of the same pH was put on top of the solution of NaCl (Tiselius, 1938), and on this a layer of cotton wool (Svensson, 1947). In Exp. 1 a continuous current of buffer was maintained within the cathode vessel from the top towards the cotton-wool pad, in addition to the compensation current through the U-tube. At the end of this run which lasted 22 hr. (with an interruption of 11 hr.), the concentration of Cl^- in the phosphate solution taken from the U-tube was less than 0.0002N, or 2% of the anion concentration of the phosphate solution used.

The relative change in migration velocity per unit concentration, K , was determined for HbO_2 , as previously described for serum albumin (Hoch, 1948), by observing the migration velocity of a boundary of a dilute solution of HbO_2 of 0.1 or 0.05 g./100 ml. simultaneously with that of a boundary of a solution of 1.15 g./100 ml. The migration velocities at given currents were determined in the usual manner at the beginning and at the end of an experiment.

The concentrations of HbO_2 were determined by photoelectric measurements of light absorption in the visible region, and those of albumin by micro-Kjeldahl.

The diffusion constant (D) was determined under the conditions of the distribution of the buffer ions as obtaining at the ascending boundary. For example, in the case of HbO_2 , a solution of 1 g./100 ml. was electrolysed till the greater part of the ascending limb was taken up by the solution forming between the HbO_2 boundary and the δ boundary. A fresh boundary was then formed by the sharpening technique of Kahn & Polson (1947), as modified by Alberty (1948), immediately below the original boundary and left to diffuse at 0° . D was determined by the area maximum height method ($A, h_{\max}$) employing from five to seven readings distributed over a period of from 1 to 20 hr. The coefficient of variation of the individual readings from a straight line plot of $(A/h_{\max})^2$ against time was 1.4%.

Observation of boundaries. The changes in the form of a boundary are most easily detected by measuring the changes in the height of the peak and this method was used throughout. The tilt of the diagonal wire was kept constant except where the change in the height was greater than could be recorded with one tilt angle. The values were then calculated to refer to a constant tilt angle.

It is important to keep the boundary in the same place if small changes in the shape of the pattern are to be observed, otherwise the defects of the optical correction of the system may interfere. While in Exp. 1, the boundary was always kept near and photographed at a chosen position in the U-tube, in Exp. 2 the readings of the heights of the peaks were corrected by linear interpolation between the values obtained at different distances from the chosen position.

RESULTS

Heterogeneity of oxyhaemoglobin preparations from red cells

In addition to the components of the normal human erythrocyte described by Stern *et al.* (1945), two components faster than the oxyhaemoglobin were

found in fresh red-cell extracts. Fig. 2*a, b* shows the ascending patterns recorded in a run with an oxyhaemoglobin solution of 1.34 g./100 ml. The two faster components are present in quantities much smaller than appeared from the relative proportions of the areas underneath the peaks, because the similarity of their mobilities with that of the main component causes an increase in their concentration ahead of the main boundary to a multiple of their original concentration. With simplifying assumptions (Hoch-Ligeti & Hoch, 1948), including the absence of specific interaction between the components, a concentration of 2.5% of the total was derived. These faster peaks could also be seen in patterns from a run with a solution of 1 g./100 ml. (from a different subject), but here their size was

the boundary after it had migrated 17 and 56 cm. respectively. The shape has hardly changed. The boundary was slightly more asymmetrical at the later stage.

In a second experiment similar results were obtained as seen in Fig. 3*b*. In this experiment no precautions had been taken to prevent the NaCl from reaching the U-tube except for the insertion of the cotton-wool pad. The steady state reached, after diffusion of the boundary had taken place overnight, gave a 17% smaller height of the peak than was observed before the boundary was left to diffuse. The migration velocity (at 12.0 ma.) was found to be 12.5% less at the end of the experiment than at the beginning, an indication of an increase in conductivity. At the end of the run the concentration

Fig. 2. Electrophoretic patterns of HbO_2 and of albumins. *a, b*, HbO_2 , 1.34 g./100 ml.; *c-f*, 1.0 g./100 ml.; 0.01 M Na_2HPO_4 , 16 V./cm.; times of migration, min. *a*, 508; *b*, 943 (distance migrated about 46 cm.); *c*, 90; *d*, 206; *e*, 360; *f*, 1205 (distance migrated about 56 cm.). *g-j* bovine serum albumin, 1.15 g./100 ml., 9.3 V./cm.; *k, m*, human serum albumin from electrophoresis of serum diluted 1:4, 10.4 V./cm.; both albumins in phosphate, pH 8, $I=0.1$; times of migration, min.: *g*, 176; *h*, 778; *j*, 1142; *l*, 540; *m*, 293, after the freshly formed boundary had been left to diffuse for 12 hr. *k*, same bovine serum albumin as in *g-j* at about 1 g./100 ml., acetate, pH 4, $I=0.02$; distance migrated 8.1 cm. All patterns are from ascending boundaries, the direction of migration is towards the left.

smaller (Fig. 2*d*). A calculation similar to that above gave a value of 1.5% of the total. It is necessary to separate these peaks sufficiently before the oxyhaemoglobin boundary can be studied.

With solutions that have been kept in the cold for 2 weeks or longer, a small peak occurs between the main peak and the slower peak (Fig. 2*c*), but in fresh preparations this is barely detectable.

The steady state

Haemoglobin. Fig. 3*a* shows a plot of the height of an oxyhaemoglobin boundary peak against the time and distance of migration. A stationary state was reached after 430 min. and it was maintained for a further 230 min. After the boundary had been left to diffuse overnight, the stationary state was reached again after migrating for about 250 min. and was then maintained for 350 min. Fig. 2*e, f* shows

of Cl^- in the supernatant phosphate solution from the ascending limb was about 0.002N; this is 20% of the anion concentration of the phosphate solution. The observed broadening of the boundary could thus be accounted for.

Serum albumins. Fig. 4*a* shows the changes in the height of the peak during 21 hr. of electrophoresis of a solution of crystallized bovine albumin. Some of the patterns are reproduced in Fig. 2*g-j*. The height of the peak diminished progressively except for a slight rise after the first period of diffusion (not recorded in the figure) and no steady state was reached. That the progressive spreading of the boundary could not have been due to a change in conductivity was ascertained by measuring the migration velocities at the beginning and at the end of the experiment. They were found to differ by less than 1%. Therefore, although the boundary peak was symmetrical and

Fig. 3. Tests for electrophoretic homogeneity, HbO_2 . For details see text.

Fig. 4a.

Fig. 4b.

Fig. 4. Tests for electrophoretic homogeneity, albumins. *a*, bovine serum albumin; *b*, human serum albumin from a patient with hypertension.

sharp during a migration over a distance considerably greater than the length of the U-tube, the slow broadening of the peak had to be interpreted as arising from heterogeneity of the preparation. Towards the end of the run the boundary had become very diffuse so that the relative changes in width due to heterogeneity were too small to produce noticeable changes in the height. The total distance migrated was 46 cm. Heterogeneity was confirmed in an electrophoresis experiment at pH 4 in which four major components were revealed (Fig. 2*k*).

measured in the absence of an electric current is used in the equations, a minimum value for the width can be derived. This was done in the case of oxyhaemoglobin. Duplicate runs gave 4.03 and 3.95×10^{-7} respectively. The oxyhaemoglobin concentrations in the adjusted solutions in Exps. 1 and 2, were $C_0 = 0.9$ and 0.63 g./100 ml., $V_0 = 7.3 \times 10^{-4}$ cm./sec. and K (for $C = 1.15$) $= 0.124$ and 0.122 (duplicate experiments). With these values the curve of Fig. 1 had been computed. Fig. 5*a* shows a tracing of the boundary in Exp. 1 for comparison. The experi-

Fig. 5. Tracings of experimental boundary patterns with superimposed theoretical curves; *a*, HbO₂, after 433 min. migration; *b*, bovine albumin after 174 min. migration.

A similar kind of heterogeneity was observed with the albumin of a serum from a subject with hypertension (Fig. 2*l*, *m* and Fig. 4*b*). After the boundary had migrated for 22 cm. a new boundary was formed by the sharpening technique (Kahn & Polson, 1947) and left to diffuse for 12 hr. On renewed electrophoresis the peak became at first narrower and higher, but after 135 min. it began to broaden again. This broadening of the peak after an initial sharpening was a clear indication that no steady state of the boundary was possible with this material. Comparison of the widths of the boundaries in Fig. 2*h*, *l* shows that the bovine albumin was heterogeneous to a greater degree than the particular human sample. The albumin concentrations in the adjusted solutions were 1 g./100 ml. in both cases.

Calculation of the width of a boundary in the steady state

Haemoglobin. From equations (5) or (10) the width of the boundary peak at a given relative height can be calculated if V_0 , K , C_0 and D' are known. It does not seem feasible to determine D' experimentally, but if the diffusion constant which can be

mental boundary peak was narrower near the top and broader near the base. The calculated minimum for the width of the boundary at half the maximum height ($0.5 h_{\max}$) then works out to 1.9×10^{-2} cm. The experimentally found widths of the peaks in the steady stage at $0.5 h_{\max}$ were 11 and 9×10^{-2} cm. respectively. These are about five times the calculated minimum values.

Albumin. The calculation of the minimum width of the peak at $0.5 h_{\max}$, using the values for the constants which apply to the conditions of the experiment with albumin, gave 3.5×10^{-2} cm. The widths of the boundary of bovine albumin found experimentally were 18 and 27×10^{-2} cm. after migrated distances of 6.6 and 11 cm. The potential gradient was 9.3 V./cm. The widths of the boundaries of serum albumin were 16.6 , 24.5 and 28×10^{-2} cm. after the boundaries had migrated 6.4 , 12.1 and 14.8 cm. The potential gradient was 10.4 V./cm. The degree of heterogeneity of the serum albumin was less than that of the bovine albumin. Fig. 5*b* shows a tracing of the boundary of Fig. 2*g* with a curve superimposed, calculated from equation (10) with a value of $\frac{V_0 K C_0}{D'}$ to give the best fit.

The test for electrophoretic homogeneity

The following procedure may be suggested. A buffer is used in which the ascending boundary is sharper than the descending and in which the migration velocity is high at the maximum current permissible. A preliminary run is made, with the ascending side towards the open electrode vessel, the other being closed (Longworth & MacInnes, 1939), until the ascending boundary has migrated 6 or 7 cm. in order to ascertain that the material gives a single boundary only, or to separate slower impurities. The current is then interrupted, a fresh boundary is formed immediately below the original boundary and the boundary is left to diffuse. If, on restarting the current, the boundary becomes sharper, it is then observed for as long a period as possible, while a steady current of buffer solution is maintained in order to keep the boundary in position. Broadening of the boundary after an initial sharpening should be ascribed to electrophoretic heterogeneity. An accidental broadening due to mechanical disturbance will be followed by sharpening if the material is homogeneous.

DISCUSSION

The width which a boundary should reach in the steady state has been calculated assuming that the only factor which tends to cause spreading of the boundary is thermal diffusion of the protein ions. In the case of oxyhaemoglobin in which a steady state of a boundary has been realized experimentally, the width of the boundary was five times this value. This discrepancy is probably, in the first place, a consequence of the neglect of the diffusion of the buffer salt ions. Only a small part of the discrepancy between the width calculated and that found experimentally can be attributed to the effects of the presence of different ionic forms since, according to Ogston (1946), the mean life of the individual forms is likely to be of the order of 10^{-14} sec. and the spreading due to this cause small compared with that due to diffusion. On the other hand, the attainment of a steady state is strong evidence for the electrophoretic homogeneity of the main component of normal human red cells. Assumption of different interconvertible forms with comparatively long mean life times may contribute to the spreading. The reactions between these forms would have to be of non-ionic type.

The characteristic deviation of the experimental shape of the boundary pattern from that of the Gaussian error curve is similar to that of the calculated curve, but this deviation is much more pronounced, particularly near the base. The type of tailing-off observed here may be explained qualitatively by the tendency of the buffer salt ions to establish a linear concentration gradient across the protein boundary.

The conditions of pronounced boundary anomalies are not only useful in that they facilitate the formation of a steady state from a diffuse boundary, but they also provide a means of concentrating constituents, which are present in traces only, simultaneously with separating them from the main component. The less the difference between their mobilities and that of the main component, the greater the degree of concentration. By this means it was possible to isolate with comparatively little effort the components which had a slightly greater mobility than oxyhaemoglobin in sufficient quantity to study the spectral absorption. Drs E. M. Jope and E. Johnson found that this material was spectroscopically indistinguishable from the bulk of the oxyhaemoglobin as regards the visible region and the Soret band.*

SUMMARY

1. The theoretical shape of the pattern of a boundary in the steady state has been calculated.
2. A steady state, indicating electrophoretic homogeneity, has been realized experimentally with the main component of normal human red-cell extracts.
3. With crystallized bovine albumin, or with the albumin in a serum from a patient with hypertension, no steady state was attained. These materials were considered to be heterogeneous, although they showed a single peak during prolonged electrophoresis.
4. A method of testing for electrophoretic homogeneity has been outlined.
5. The presence in red-cell extracts of two electrophoretic components, not hitherto described, has been demonstrated.

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The Irreversibility of the Deamination of Threonine in the Rabbit and Rat

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It has been known for some time from perfusion and growth experiments with mammals that many amino-acids are deaminated to keto acids and that these can be reconverted to the original amino-acids. Thus, the fact that many essential amino-acids can be replaced in the diet by the D-enantiomorphs or by the corresponding hydroxy or keto acids indicates that deamination and reamination occur readily. However, the extent and the speed of such reactions was not fully realized until studies with isotopic nitrogen were initiated in the field of intermediary metabolism (for reviews see Schoenheimer, 1942; Schoenheimer & Rittenberg, 1940). It was shown that, after feeding to rats ammonium salts, glycine, L- or D-leucine or L-tyrosine labelled with ^{15}N , almost all the amino-acids isolated from the proteins of different tissues contained some excess ^{15}N . The amide nitrogen and the amino nitrogen of glutamic and aspartic acids always had the largest concentrations of the isotope, but some 'essential' amino-acids such as histidine and leucine also exchanged reversibly their α -amino nitrogen with that of other amino-acids or with ammonia. For other essential amino-acids such as tryptophan, methionine and phenylalanine, such a reversible deamination and reamination has not been demonstrated by the isotope method, but positive evidence for the occurrence of such reactions has been obtained from growth experiments using D-amino-acids and also related keto and hydroxy acids (for reviews, see Rose, 1938; Jackson & Chandler, 1939; Neuberger, 1948). Negative evidence from such nutritional experiments is not conclusive. Thus D-valine, D-leucine and D-isoleucine are not available for growth in the

rat (Rose, 1938), and similar results have been reported for the mouse (Bauer & Berg, 1943). The chick can utilize D-leucine, but not D-isoleucine or D-valine (Grau & Peterson, 1946). On the other hand, Schoenheimer, Ratner & Rittenberg (1939), using L-leucine labelled with ^{15}N and deuterium, showed that this amino-acid exchanges its nitrogen with that of other amino-acids. Later, the inversion of D- to L-leucine in the rat was clearly demonstrated by the method of double labelling (Ratner, Schoenheimer & Rittenberg, 1940). These findings and the observation of Rose (1938) that the three keto acids corresponding to leucine, valine and isoleucine support growth indicate that these three essential aliphatic amino-acids yield nitrogen to, and accept nitrogen from, other amino-acids. The negative results of growth experiments with the D-amino-acids may be explained by assuming that the rate of deamination is too slow for the requirements of growth.

The only amino-acid which had been isolated by the Columbia workers and which never contained any excess ^{15}N was lysine (Foster, Schoenheimer & Rittenberg, 1939). It had been shown earlier (Foster, Rittenberg & Schoenheimer, 1938) that lysine, unlike other amino-acids, does not take up deuterium from the body water. By the method of double labelling (deuterium and ^{15}N) it was demonstrated that the products of deamination of lysine cannot be aminated again to form lysine (Weissman & Schoenheimer, 1941). This conclusion agrees fully with the observations that neither D-lysine (Berg, 1936) nor any known derivative of lysine, with the exception of ϵ -N-methyllysine and ϵ -N-acetyllysine