5. Under the experimental conditions used, chloramphenicol reduces the synthesis of deoxy-ribonucleic acid.

6. Deoxyribonucleic acid increases the activity of incorporation factor in promoting the incorporation of adenine into ribonucleic acid.

7. A number of inhibitors, including 8-hydroxy-quinoline and derivatives of benzimidazole, inhibit the incorporation of adenine or guanine to the same extent as they inhibit the incorporation of glycine.

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Electrophoretic Behaviour of Human Serum Albumin at pH 4

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The separation of more than one moving boundary during electrophoresis of horse and human serum albumins in solutions of low ionic strength near pH 4, when first observed by Luetscher (1939), was assumed to be due to a 'true' heterogeneity of the albumins, i.e. to the existence of protein molecules differing intrinsically in electrochemical properties. Saifer & Corey (1954, 1955, 1956) were inclined to favour this view after obtaining multiple peaks with several serum albumins, ovalbumin and γ -globulin in buffer solutions of low ionic strength. On the other hand, Aoki & Foster (1957a, b) interpreted the results of their electrophoretic experiments with bovine serum albumin at low pH, as well as those of Phelps & Cann (1956), in terms of certain equilibria prevailing in the solutions. Since it is of considerable interest to establish whether or not the serum albumins exhibit a 'true' electrophoretic heterogeneity, several lines of approach have been taken in the present work.

A study has been made of the variations produced in the electrophoretic pattern of human serum albumin by various organic-buffer anions at constant pH, ionic strength and protein concentration. In some cases material isolated from the fastest-moving layers has been run again after further dialysis. Experiments have also been made with albumin labelled with radioactive iodine, and comparing specific activity and schlieren curves. The evidence accumulated does not support the

case for 'true' electrophoretic heterogeneity, but favours the equilibrium hypothesis, a conclusion similar to that reached recently by Phelps & Cann (1957) in connexion with the electrophoresis of bovine serum albumin.

EXPERIMENTAL

Protein. The protein used in this work was the human serum albumin used previously (Charlwood, 1954), and distinguished by the designation A. For most experiments, solutions of rather more than 2% protein were dialysed against changes of buffer for several days. After the refractive-index increment had been measured at 578 m μ by means of a dipping refractometer, the protein solution was diluted to 2.00% (w/v), assuming a specific refractive increment of 0.00184 (Charlwood, 1957).

Buffers. These were mainly of ionic strength 0.02. The pH was adjusted to 4.00 ± 0.05 , as measured at room temperature (20°) with a Marconi instrument equipped with a glass electrode, and referred to 0.05M-potassium hydrogen phthalate as standard (pH 4.00).

Electrophoresis experiments. These were done in the Tiselius apparatus (mostly at 2°), with the diagonal-schlieren optical system with a mercury-vapour lamp from which light of wavelength 546 m μ was isolated. The current used was usually not more than 4 ma in the standard cell, corresponding to a maximum voltage gradient of about 6.7 v/cm. in the buffer. Lower currents were essential in the runs at 20° (Johnson & Shooter, 1948). Only ascending boundaries were recorded, as the descending ones were very diffuse and anomalous under the conditions used. When extended runs were required, a photoelectric device was

used to control the position of the leading peak (Warren & Charlwood, 1953). As the faster-moving material isolated from a single run was insufficient for a subsequent run, the product of three similar runs was combined and concentrated by pressure dialysis before being used in the micro (3 ml.) cell.

Radioactive studies. A typical experiment with radioactive material was carried out as follows. A sample of the albumin was dissolved in buffer (1.28% glycine, 0.18% sodium hydroxide, 0.25% sodium chloride; pH 9.3) and labelled with ^{125}I by the jet method (McFarlane, 1956) to the extent of about one atom of total iodine/molecule, and a specific activity of 5 mc/g. A portion of iodinated protein was mixed with several times its weight of untreated albumin dissolved in lactate buffer, and thoroughly dialysed against this buffer. It was submitted to electrophoresis in the standard cell at a concentration of about 2% albumin, as in Fig. 1 (c), but for a longer time (about 3 hr.). The contents of the section containing the faster boundaries were isolated, mixed with more untreated albumin to bring the total protein concentration back to about 2%, and further dialysed. The next stage was a run in the flow-through type of cell (Kekwick, Lyttleton, Brewer & Dreblow, 1951), under the same conditions, and for about 2½ hr. The column of solution, slowly expressed from the exit capillary, was collected as 3 drop fractions (~0.15 ml.). These were each diluted with 3 ml. of buffer (or water with oxalate solutions), relative activities determined in a scintillation counter and protein concentrations measured in a Beckman DU spectrophotometer at 280 m μ wavelength. At this wavelength buffer absorption (except for oxalate of ionic strength 0.02) was negligible. Some runs with iodinated albumin were done in the flow-through cell without any preliminary separation of the faster layers. Specific-activity curves were correlated with the schlieren patterns by integrating the latter to different ordinates and comparing with the concentration-height curves constructed from the optical-density figures.

Sedimentation experiments. Solutions containing 1.5% of albumin in buffers of ionic strength 0.02 (pH 4) were examined in a Spinco ultracentrifuge operated under the usual conditions (Charlwood, 1954). Acetate, lactate and oxalate were used in these experiments.

RESULTS

The different types of pattern given in the presence of the various anions are illustrated in Fig. 1. Patterns for mandelate and salicylate are not included as they seemed to give rise to convections or precipitation in the cell. Whereas formate and barbiturate gave patterns of the lactate type, propionate, malate and succinate gave rise to the acetate type. In most of the buffers (Fig. 1) the leading peak was followed by a smaller one, which was absent in phthalate. This peak was also not visible in citrate if the pH were slightly higher (4.10). A third moving peak, also travelling towards the cathode, was resolved in lactate, formate and barbiturate (and also detected in citrate), and in citrate, oxalate and phthalate there was an additional boundary moving towards the

anode. The boundaries which remained approximately stationary were probably of the δ type (Tiselius & Kabat, 1939). Continuation of the runs produced generally some further resolution, e.g. a slow-moving peak separating from the stationary one in acetate, and further subdivision of the leading peak in acetate, citrate, etc. (Fig. 2). These effects,

Fig. 1. Electrophoretic patterns in various buffers ($I=0.02$) at protein concentration of 2.00% (w/v). (Approximate positions of initial boundaries are indicated by arrows.) Temperature 2.0°, except in (b). (a) Acetate (pH 4.02), 60 min. at 6.5 v/cm.; (b) acetate (pH 4.04), 163 min. at 1.36 v/cm.; and 20.0°; (c) lactate (pH 3.97), 74 min. at 6.7 v/cm.; (d) oxalate (pH 3.99), 139 min. at 4.2 v/cm.; (e) citrate (pH 4.02), 86 min. at 6.3 v/cm.; (f) phthalate (pH 4.00), 279 min. at 5.0 v/cm. Patterns similar to (a) were given with propionate, malate and succinate, similar to (c) with formate and barbiturate.

Fig. 2. Electrophoretic patterns ($I=0.02$) after further electrophoresis. Protein concentration, 2.00% (w/v). Migration was to the left. Temperature 2.0°. (a) Acetate (pH 4.02), 180 min. at 6.5 v/cm.; (b) acetate (pH 4.02), 300 min. at 6.5 v/cm.; (c) citrate (pH 4.02), 271 min. at 6.3 v/cm. [Slow-moving peaks are no longer visible in (b) and (c).]

which were reproducible, did not appear to be associated with convections.

Incorporation of 0.02M-sodium chloride in the acetate and lactate buffers did not produce any marked effects on the pattern (Fig. 3*a, c*), but at the same total ionic strength (i.e. 0.04) and different proportions of acetate and chloride (0.036M-sodium chloride, acetate of ionic strength 0.004) an extra moving peak occurred (Fig. 3*b*).

Variation of the protein concentration produced some alteration in the relative area distribution under the peaks, especially a reduction in size of the stationary boundary (Figs. 1*c, 5a*), but there was no change in the number of peaks (cf. Saifer & Corey, 1955).

The effect of using a temperature of 20° instead of 2° was to give rather more diffuse patterns, as might be expected, but otherwise little difference could be observed (e.g. Fig. 1*a, b*).

Fig. 3. Electrophoretic patterns at different buffer concentrations and protein concentration of 2.00% (w/v). Migration was to the left. Temperature 2.0°. (a) Acetate ($I=0.02$) + chloride ($I=0.02$), pH 4.00, 459 min. at 1.70 v/cm.; (b) acetate ($I=0.004$) + chloride ($I=0.036$), pH 3.95, 278 min. at 1.76 v/cm.; (c) lactate ($I=0.02$) + chloride ($I=0.02$), pH 4.03, 477 min. at 1.40 v/cm.; (d) lactate ($I=0.04$), pH 4.00, 139 min. at 3.7 v/cm.

Fig. 4. Electrophoretic patterns in lactate buffer (pH 3.97, $I=0.02$) after (a) 171 min. at 6.7 v/cm. and (b) 153 min. at 5.0 v/cm. Migration was to the left. Dotted line indicates the level of exit capillary in (b). Protein concentration, 2.0% (w/v). Temperature 2.0°.

The course of a typical re-running experiment is illustrated in Figs. 4 (a) and 5 (b). Material separated from the left-hand section in runs carried to the stage shown in Fig. 4 (a), after concentration by pressure dialysis, gave the pattern shown in Fig. 5 (b). The corresponding pattern obtained with unfractionated albumin (Fig. 5c) was indistinguishable from it.

The stages at which the faster layers were separated, and the radioactive fractions run off, are illustrated in Fig. 4 (a), (b). Curves obtained from runs in phosphate and lactate buffer in flow-through experiments are shown in Fig. 6. Curves of optical density (not shown) were consistent with ones derived from the schlieren curves by integration, but the former were rather flatter, showing that there was a small amount of mixing during the passage of the column of solution through the exit tubes from the cell. The specific activity curve in phosphate buffer (Fig. 6a) was linear, but in lactate it was composite, corresponding with the more complex refractive-index gradient curve. It is important, however, that the curves in Fig. 6 (b), (c) are identical in type, indicating that some of the protein which moved in the leading peaks in the first experiment had been converted into a form of lower mobility, whether by ion binding, change in molecular configuration or some other process.

In the ultracentrifuge runs only a single peak was observed (Fig. 7). Although it had some

Fig. 5. Electrophoretic patterns in lactate ($I=0.02$, pH 3.96). Migration was to the left. Temperature 2.0°. (a) 0.5% (w/v) protein after 150 min. at 3.4 v/cm.; (b) 1.35% (w/v) protein after 91 min. at 4.2 v/cm. in the micro-cell. Re-dialysed from isolated leading layers. (c) 1.36% (w/v) protein after 103 min. at 4.2 v/cm. in the micro-cell.

asymmetry, which might be expected under the conditions used, there was no evidence for the existence of aggregates.

DISCUSSION

Substitution of chloride for part of the buffer anion (Phelps & Cann, 1956) in these systems has the disadvantage of increasing the complexity of the system, and simultaneously altering the buffer capacity. It was hoped that, by the use of a variety of organic acids and their sodium salts, it would be possible to examine systems of comparable buffer capacity in which the main effects were clearly attributable to the specific ion effects alone. However, approximate calculation showed that there

were considerable individual variations in buffer capacity (Table 1) when pH and ionic strength were standardized.

A comparison of Table 1 with Fig. 1 shows that, for the series of univalent buffers, the complexity of the electrophoretic pattern increased as buffer capacity decreased. A similar tendency was observed among the aliphatic dibasic acids. These results may be compared with the effect of chloride on patterns obtained in acetate. Where the buffer capacity was undiminished (Fig. 3a) the pattern was slightly more diffuse, but when the buffer capacity was reduced (Fig. 3b) an extra peak appeared. If the buffer capacity, relative to the concentration of protein present, were of great significance, a reduction of protein concentration (or an increase in buffer concentration) might alter the number of peaks obtained. In lactate buffer, at least, this did not occur (Figs. 3d, 5a). The actual differences of pH between various layers, even under conditions similar to those used here, have been found experimentally to be relatively small (E. F. Woods, personal communication).

The unfavourable ratio of salt to protein concentration is likely to result in large superimposed gradients of salt or protein at the boundaries. This is illustrated by the marked reduction in the size of the stationary boundary as the protein concentration is lowered (Figs. 1c, 5a). Measurements of relative peak areas or attempts to estimate mobilities would therefore be of limited value,

Fig. 6. Specific-activity (●) and refractive-index gradient curves for iodinated albumin in (a) phosphate ($I=0.2$, pH 7.96) after 341 min. at 3.35 v/cm.; (b) lactate ($I=0.02$, pH 4.00) after 153 min. at 5.0 v/cm.; (c) lactate ($I=0.02$, pH 3.97) after 145 min. at 5.0 v/cm. In (c) the protein consisted of leading layers separated by preliminary electrophoresis of iodinated albumin, together with untreated carrier albumin. Temperature 2° in all cases.

Fig. 7. Ultracentrifuge pattern obtained with 1.5% (w/v) albumin in oxalate ($I=0.02$, pH 4.00) at 21.2°. Sedimentation (right to left) for 131 min. at 59 780 rev./min.

Table 1. Relative buffer capacities at ionic strength 0.02, pH 4.0

Propionate	1.00	Succinate	0.68
Acetate	0.95	Malate	0.46
Barbiturate	0.56	Oxalate	0.15
Lactate	0.47	Phthalate	0.18
Formate	0.38	Citrate	0.25

unless a reliable extrapolation to zero protein concentration could be made.

The effects observed, whether or not partly dependent on buffer capacity, must involve specific interaction between buffer anions and protein. This could involve albumin molecules with inherently different characteristics, or alternatively might arise from a population normally homogeneous, if binding of anions involves a statistical distribution (Klotz, 1953). In all the solutions the number of univalent buffer ions present is large (~ 50 /albumin molecule) but bivalent or tervalent ions are present in much smaller numbers. A dynamic equilibrium, even if rapidly attained, might still result in the separation of distinct peaks, as has been demonstrated theoretically in a formally similar case in the ultracentrifuge (Gilbert & Jenkins, 1956). These authors considered an instantaneous equilibrium of the type $A + B \rightleftharpoons C$, where the three species have different sedimentation coefficients. Diffusion was neglected to enable the differential equations to be solved analytically, and curves were computed to show that more than one maximum could still arise in the schlieren pattern. Gilbert & Jenkins (1956) stated that, according to their theory, the boundary keeps its essential shape, and merely stretches out with time. If equilibrium were not attained instantaneously, we should expect the characteristics of the pattern to be intermediate between this and the normal type given by three species sedimenting independently. The complexity of the schlieren pattern could conceivably increase with time if the equilibrium required a moderate time to be established. The splitting of the leading peak at longer times in the present experiments may be such an example. The minor effects of temperature alterations are not incompatible with ion-binding, which is often not greatly affected by differences of temperature (Klotz, 1953). The more strongly an anion is bound at pH 4, the lower becomes the net charge (and most probably the mobility) of the protein. Very strong binding might produce a reversal of the sign of the charge when the protein is not too far from the isoelectric point. Of the three buffers in which a boundary was detected moving towards the cathode, one at least (phthalate) is known to bind strongly to albumin (Klotz & Urquhart, 1949). The slow rate of movement of the main peak in phthalate, compared with most of the other buffers of ionic strength 0.02, would also be consistent with strong binding. The leading boundary, and the smaller one observed adjacent to it in most buffers, may correspond with the two interconvertible forms of albumin postulated by Aoki & Foster (1957*a, b*). This is supported by the disappearance of the smaller one in citrate when the pH is slightly higher.

The re-running experiments clearly demonstrated that the material which migrated in the leading boundaries was redistributed after dialysis. In contrast with this, Saifer & Corey (1956) claimed that the fastest layer separated from human γ -globulin could be re-run as a single peak, although their second run was done at a much lower concentration.

Iodinated albumin helped to confirm the conclusions drawn from the re-running experiments, but the specific-activity curves were not susceptible of a simple interpretation. The sharp rise of specific activity through the two leading peaks in Fig. 6 (*b, c*) is probably due to an increase in electrophoretic mobility of albumin molecules when iodine substituted, a suggestion supported by other evidence (Charlwood & Gordon, 1958). The variation in specific activity through the third peak may be due to a similar mobility effect, but it could also be due, partly or wholly, to different anion-binding characteristics of substituted and non-substituted albumin molecules. The plateau region of the activity curve corresponding with the stationary boundary supports the contention that this boundary represents nearly a pure dilution effect (δ boundary).

In earlier work (Charlwood, 1954) no differences were detectable in the diffusion and sedimentation behaviour of whole albumin and that in the leading boundary in acetate buffer, ionic strength 0.02, pH 4.0. This is consistent with the ultracentrifugal evidence presented here against the hypothesis that aggregation might be a contributing factor.

It is concluded that the behaviour of human albumin at pH 4 does not afford any evidence of a 'true' electrophoretic heterogeneity, although it does not prove that albumin does not contain families of molecules differing fundamentally in some other physical properties (cf. the solubility studies of Derrien, 1947). The observed patterns must be produced by anion interaction, which may also account for the apparent heterogeneity of human albumin on prolonged running in phosphate (Blix, Tiselius & Svensson, 1941; Hoch & Morris, 1945) and in veronal buffer (Charlwood, 1954).

SUMMARY

1. Electrophoretic patterns have been obtained for human albumin at pH 4 and low ionic strength (0.02) in the presence of various organic anions.
2. The faster-moving layers in some instances were removed and run again after further dialysis.
3. Albumin labelled with radioactive iodine was used in experiments designed to show the variations of specific activity through the different moving boundaries.

4. It is concluded that all observations are to be explained in terms of protein-ion interactions rather than true protein heterogeneity.

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Paper Chromatography of Saturated and Unsaturated Fatty Acids

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During an investigation of fat metabolism in the fungus *Trichoderma viride* it became desirable, in order to avoid culturing excessive quantities of mycelium, to find a method of separating micro-quantities of natural fatty acids. Such a method would also be useful for radioautographic purposes. In this communication the behaviour of over 40 pure fatty acids on several reversed-phase paper-chromatographic systems is examined: the systems are liquid paraffin/aqueous acetone (adapted from a column-chromatographic method, see Howard & Martin, 1950; Silk & Hahn, 1954; Crombie, Comber & Boatman, 1955), liquid paraffin/aqueous acetic acid (a system resembling that of Kaufmann & Nitsch, 1954; Wagner, Abisch & Bernhard, 1955; Seher, 1956; Leibnitz *et al.* 1957), castor oil/aqueous acetone (adapted from the column system of Savary & Desnuelle, 1953; Matic, 1956), castor oil/aqueous acetic acid, and polythene/aqueous acetone (adapted from the column method of Green, Howitt & Preston, 1955).

METHODS AND MATERIALS

Impregnation of papers

(1) *Liquid paraffin*. Whatman no. 1 chromatography papers (13 cm. × 45 cm.) were immersed in a solution of

liquid paraffin (Nujol) (35 ml.) in benzene (65 ml.), blotted (10 min.) under an evenly distributed 18 lb. weight and dried by hanging in air at room temperature. Provided that the same grade of blotting paper is used, a constant weight of paraffin (25%) remains on each paper. It is important to avoid excess of paraffin since this results in loss of fatty acid during formation of the copper salts (for full details see below under 'Quantitative paper chromatography of fatty acids'). After removal of the top (11 cm.) and bottom (4 cm.) portions, each paper was cut into standard 30 cm. × 2 cm. lengths ('top' and 'bottom' refers to a paper hung vertically with the direction of machining pointing upwards).

(2) *Castor oil*. A solution of castor oil (B.P. grade) in ethyl ether was shaken three times with 4% (w/v) aq. K_2CO_3 to remove free fatty acids. After the solution had been washed three times with water and dried (Na_2SO_4) and the solvent evaporated, the neutral oil (35 ml.) was dissolved in benzene (65 ml.) and this solution used for impregnating papers as in (1) above.

(3) *Polythene*. Polythene powder (Schori Metallising Process Ltd.) was dissolved in xylene [3 or 6% (w/v) final solution] at 60° and papers impregnated with the hot solution. They were air-dried at 20° until solvent-free.

Application of fatty acids to the paper

Pure fatty acids or mixtures, dissolved in $CHCl_3$ (approx. 6 mg./ml.), were delivered (1–5 μ l. at a time, total volume 10–20 μ l.) on the starting line (5 cm. from the upper edge