The method has been applied to hydrolysates of human γ -globulin and of a protein from the surface antigen of *Pseudomonas aeruginosa* L2 (Mead & van den Ende, 1953) and also to concentrated normal urine. The globulin hydrolysate gave spots corresponding to aspartic and glutamic acids, cystine, serine, threonine, tyrosine, glycine, alanine, valine, leucine (isoleucine), phenylalanine, proline, histidine, lysine and arginine. Spots at the positions taken by 13 of the common amino acids were detectable on the ionophoretogram given by the antigen-derived protein. The urine concentrate served to indicate that the method may be applied directly to solutions rich in dissolved solids. Taurine, glycine, serine, asparagine, methionine, alanine and histidine were tentatively identified.

Two-dimensional ionophoresis should give better separation than one-dimensional in all cases where change of pH affects the relative as well as the absolute rate of migration of the substances to be analysed. In practice, however, difficulties in finding sufficiently volatile buffer components may be a limiting factor. Mixtures of proteins are an obvious field for experiment also lipoprotein-polysaccharide complexes such as some bacterial antigens for at least one of which (that of *P. aeruginosa* strain L2) the excellent method of detection on paper due to Rydon & Smith (1952) may be used.

SUMMARY

1. In a new apparatus for two-dimensional paper ionophoresis, the pH of the buffer solution in the paper is changed, after ionophoresis in one dimension, by exposure to the vapour from a solution exerting a suitable and substantially constant partial pressure of ammonia.

2. A method for determining the flow of electrolyte at different points on the paper is proposed. The results of applying it in the new apparatus are discussed.

3. The following amino acids are separated qualitatively by the new apparatus: glutamic and aspartic acids, cystine, glycine, alanine, valine, leucine, phenylalanine, serine, threonine, tyrosine, hydroxyproline, proline, histidine, lysine and arginine. Leucine and isoleucine are not separated. The developed ionophoretogram is ready within nine hours of starting an experiment.

I am grateful to Professor M. van den Ende for encouragement and to Dr A. Polson for many helpful discussions. The apparatus was constructed by Mr W. H. Borret without whose skilful workmanship this investigation could not have been carried out.

This work was supported by grants from the South African Council for Scientific and Industrial Research, and the Nkana-Kitwe and Chingola Poliomyelitis Research Funds.

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Separation of Neutral Proteins on Ion-Exchange Resins

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(Received 30 September 1954)

In recent years, chromatographic procedures using ion-exchange resins have been applied to the separation of a large number of substances, but their successful application to the separation of proteins has been confined mainly to basic proteins of low molecular weight, such as cytochrome c (Paleus &

Nielands, 1950) ribonuclease (Hirs, Moore & Stein, 1953) lysozyme (Tallan & Stein, 1953) and chymotrypsinogen α (Hirs, 1953). The ion-exchange resin used in the above studies was the carboxylic cation exchanger, Amberlite IRC-50. Columns of this resin have also been used to purify the active substances from a preparation of adrenotropic hormone (Dixon, Moore, Stack-Dunne & Young, 1951).

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A general chromatographic method for the separation of proteins, without denaturation, would be an extremely useful tool for the preparation of small amounts of a purified protein as well as a sensitive technique for examining the homogeneity of a particular protein preparation. The main object of the present work was to develop a chromatographic method for the separation of neutral proteins. Since coloured substances offer advantages in chromatography, the neutral proteins chosen were a group of haemoglobins. The resin used was Amberlite IRC-50.

In selecting conditions for the separation of the haemoglobins, we were guided by results obtained with the basic protein, cytochrome *c*. The effect of cation concentration and pH of buffer on the adsorption of cytochrome *c* on IRC-50 has been studied in some detail and the information gained has been used to predict conditions for the separation of the haemoglobins.

The present communication presents the results of the detailed study of the behaviour of cytochrome *c* on columns of IRC-50 and the successful separation of some haemoglobins. Preliminary accounts of this work have been published previously (Boardman & Partridge, 1953, 1954).

MATERIALS

Ion-exchange resins. The resin used in most of the experiments was a fine grade form (XE-64) of the carboxylic acid cation exchanger, Amberlite IRC-50 (obtainable from Charles Lennig and Co., London). Material in the 120 to 250-mesh/in. range (resin *A*) was obtained by removing the fines. This was done by suspending the resin in water, allowing it to settle for 30 min. and removing the supernatant suspension. This process was repeated several times until the supernatant was clear after settling for 30 min. The resin was cycled twice through the sodium form by alternate treatments with 2N-NaOH and 2N-HCl, the resin being washed with distilled water between each treatment. The resin was finally converted into its sodium form and kept under 2N-NaOH until required. In order to study the effect of variations in the resin on the adsorption of cytochrome *c*, three different batches of IRC-50 were chosen and also a methacrylic acid resin, Zeo-Carb 226 (supplied by Permutit Ltd., London). Resin *B* was from the same batch as resin *A*, but of smaller particle size (250 to 400-mesh/in.). Resins *C* and *D* were different batches of IRC-50, supplied in the form of large spherical beads. These beads were ground in a laboratory hammer mill and graded by sieving (100 to 150-mesh/in.). Resin *E* was a highly cross-linked form of Zeo-Carb 226, which had been ground and graded (100 to 150-mesh/in.).

Cytochrome *c*. This was prepared from horse-heart muscle by the method of Keilin & Hartree (1945). This method of preparation gives a cytochrome *c* with an iron content of 0.34%. A 1% solution was dialysed against 0.5% NaCl and stored at -20° .

Carbon monoxide haemoglobins. The early experiments at 25° were carried out with samples of carbon monoxide (CO)

haemoglobins which had been prepared by Dr Adair. These samples had been stored for some time at -20° . After thawing slowly, they were filtered through no. 50 Whatman filter papers using Celite as a filter aid. Later experiments were carried out with samples of bovine, sheep and sheep foetal CO haemoglobins, which had been freshly prepared by method 2 of Adair & Adair (1934). The stromata were removed by filtration through no. 50 Whatman papers with the aid of Celite.

Bovine methaemoglobin. This was prepared by mixing bovine CO haemoglobin with a $K_3Fe(CN)_6$ solution, allowing to stand for 30 min. and dialysing thoroughly.

PROCEDURES

Preparation and operation of columns. The purified resin was suspended in the buffer used for developing the chromatogram, allowed to settle and the supernatant removed. This process was repeated until the pH of the supernatant fell to within 1 or 2 pH units of the pH of the buffer. The resin was then suspended in about 2 vol. of the buffer, and the resulting slurry poured into the chromatographic tube in small portions at a time (Moore & Stein, 1951). Each portion was allowed to settle under gravity. The chromatographic tubes used in most of the experiments had an internal diameter of 0.9 cm., the resin being supported on a coarse sintered glass disk which had been sealed into the tube. The experiments with cytochrome *c* and the initial experiments with the CO haemoglobins were carried out at 25° . The chromatographic tubes were jacketed to permit temperature control by circulation of water from a constant-temperature water bath. The experiments with the haemoglobin were carried out at 2° in a constant-temperature cold room.

A separating funnel containing the developing buffer was connected to the chromatographic tube with polyethylene tubing and buffer was passed through the resin column at a flow rate of approximately 30 ml./hr. for at least 24 hr., or until the pH of the effluent was identical with the pH of the inflowing buffer. For the experiments at 2° , this equilibration was carried out in the cold room. For the experiments at 25° , the circulation of water from the thermostat bath was started at least 1 hr. before the addition of the protein sample.

After the excess buffer had drained into the column, the sample of protein was added by a pipette with a bent tip to direct the stream against the side of the tube. The protein had previously been dialysed against the developing buffer. 1.0 ml. samples of cytochrome *c* were chromatographed and 0.2 ml. samples of the haemoglobins. The amount of protein varied from 3 to 6 mg. The protein solution was allowed to drain into the resin and then the walls of the tube were washed with a little developing buffer, which was also allowed to drain into the resin. The time taken for the sample to drain into the column was 0.5–0.75 hr., a flow rate of this order being obtained by immersing the lower end of the chromatographic tube in a beaker of water.

The tube above the resin was completely filled with buffer and connected to the separating funnel. The rate of flow of buffer through the column was 1–1.5 ml./hr. in the cytochrome *c* experiments and 0.5 ml./hr. in the haemoglobin experiments. The effluent from the column was collected in 1.0 ml. (cytochrome *c*) or 0.2 ml. fractions (haemoglobins), using a time-operated or a drop-counting fraction changer.

In many experiments the volume of the fractions was checked by weighing.

Buffers. For the experiments with cytochrome *c*, sodium or ammonium phosphate buffers were used. The effect on the elution volume at pH 7.0 of reducing the phosphate concentration of the buffer to 0.04M and adding (a) NaCl or (b) Na_2SO_4 to bring the Na^+ concentration to 0.34 g. ions/l. was also studied.

0.1M Sodium citrate buffers of the following composition were used to elute the haemoglobins. 21.0 g. A.R. citric acid, 10.0 g. A.R. NaOH; solution made to approximately 950 ml.; N-NaOH was added until the desired pH was obtained, then A.R. NaCl to bring the Na^+ concentration to 0.34 g. ions/l. and the solution made up to 1 l. In the haemoglobin work, a concentrated citrate buffer was also used; this contained 220 g. citric acid and 126 g. NaOH/l. (pH 6.5, Na^+ concentration 3.2 g. ions/l.).

Analysis of effluent. Cytochrome *c* in the effluent was determined photometrically at 550 $\text{m}\mu$. using a Hilger Uvispec spectrophotometer. Before measurement each fraction was diluted with 3.0 ml. of distilled water and the cytochrome *c* reduced with a trace of sodium dithionite.

Haemoglobin also was determined by a photometric method. The positions of the absorption maxima and the corresponding millimolar extinction coefficients agreed well with those reported in the literature (Lemberg & Legg, 1949). The optical density of a CO haemoglobin solution would normally be measured at a wavelength corresponding to one of the absorption maxima, i.e. at 418 $\text{m}\mu$. for dilute solutions (1–100 $\mu\text{g./ml.}$) and at 538 or 567 $\text{m}\mu$. for more concentrated solutions. The effluent solution from the column, after dilution, were usually in the lower concentration range. However, if the optical densities were measured at 418 $\text{m}\mu$, any formation of methaemoglobin during the running of a chromatogram would lead to a serious error in the determination of the total haemoglobin content. An absorption maximum for methaemoglobin occurs at 405 $\text{m}\mu$. and the spectrophotometric curves of CO haemoglobin and methaemoglobin intersect at a wavelength of 412 $\text{m}\mu$. The total haemoglobin content of the solutions was therefore measured at 412 $\text{m}\mu$, but it should be observed that any error in the wavelength setting at 412 $\text{m}\mu$. has a much greater effect on the optical density of a CO haemoglobin solution than a similar error at 418 $\text{m}\mu$.

Crystallization of bovine CO haemoglobin from the column. 50 mg. of bovine CO haemoglobin were chromatographed on a column of IRC-50, 5.0 $\times$ 3.0 cm., with citrate buffer of pH 5.81 and Na^+ concentration, 0.34 g. ions/l. The effluent was collected in 2 ml. fractions and the fractions containing a reasonably high concentration of CO haemoglobin were mixed and the resulting solution was dialysed under pressure against 0.67 saturated $(\text{NH}_4)_2\text{SO}_4$ until the CO haemoglobin content was approximately 2%. A small amount of the solution was placed on a watchglass and allowed to stand in a cold room at 0°. Crystals of CO haemoglobin appeared overnight.

Adsorption experiments with lysine. For comparison with the experiments with cytochrome *c*, a study was made of the effect of pH on the adsorption on IRC-50 of the basic amino acid, lysine. Adsorption was carried out from buffer solutions of sodium ion concentration 0.34 g. ions/l. The buffer system depended on the pH; from pH 3 to 6, it was citrate, from pH 6 to 8, phosphate and from pH 9 to 10,

borate. The dry resin:solution ratio was approximately 1:3.6.

Procedure. The purified resin was washed thoroughly with water and dried at 105°. A sample of dry resin (0.3 g.) was weighed into a test tube and shaken with 10–15 ml. buffer. The resin was allowed to settle and the supernatant removed with a pipette. The process was repeated until the supernatant was of the same pH as the buffer. The tube was then reweighed to give the weight of the wet resin. 1.0 ml. of a solution containing 5 mg. lysine was added, the tube shaken and allowed to stand for 3 hr. with occasional shaking. A sample (0.2 ml.) of the supernatant was withdrawn, diluted to 1 ml. and adjusted to pH 5. The concentration of lysine was determined by the photometric ninhydrin method of Moore & Stein (1948).

Calculations. In order to calculate the amount of lysine adsorbed by the resin, it was necessary to know the volume of liquid held between the resin particles. This was determined by a centrifuge method (Pepper, Reichenberg & Hale, 1952). It was found that the total amount of liquid taken up by the resin (including the liquid between the particles of resin) was dependent on the pH of the buffer, but the amount of liquid removed by centrifuging remained almost constant (1.8 ml./g. dry resin). The volume of liquid in the resin phase was taken as equal to the total amount of liquid taken up by the resin minus the volume of liquid held between the resin particles. The volume of liquid in the resin phase was dependent on the pH: at pH 7.0 and a Na^+ concentration of 0.3 g. ions/l., the volume was 1.8 ml./g. dry resin and at pH 3.1 it was 0.8 ml.

As shown by Hale & Reichenberg (1949) the ionization of the resin is completely suppressed at pH 3 and the resin does not exhibit its full capacity for Na^+ (9.2 m-equiv./g.) below pH 8 or 9 (see Fig. 2). At pH 7.0 and a Na^+ concentration in solution of 0.3, the capacity of the resin for Na^+ is approximately 7 m-equiv./g. Thus an uptake of 7 m-equiv. of Na^+ corresponds to an increase of 1.0 ml. in the amount of water taken up by the resin. This corresponds to 8 m-equiv. of water for each milliequivalent of Na^+ adsorbed. The volume of liquid in the resin phase, at a particular pH and Na^+ concentration in solution, can be calculated from the titration curve of the resin if we assume that this volume is proportional to the uptake of Na^+ by the resin.

Volume of liquid in resin phase/g. dry resin

$$= 0.8 + \left[\frac{\text{capacity of resin}}{7} \times 1.0 \right].$$

In the adsorption experiments, the volume of the ambient phase was obtained by subtracting the resin phase volume from the total amount of water in the system. The amount of lysine adsorbed by the resin was the difference between the amount added and the amount remaining in the ambient phase after equilibration.

Total volume of effluent ($V + V_e$). This is equal to the total volume of liquid which flows from the column from the time of application of the solute until the maximum concentration of solute emerges from the column.

Hold-up volume (V_e). This is the volume of solvent which is held between the resin particles.

Elution volume (V). This is equal to the total volume of effluent minus the hold-up volume.

R_F value, defined as $V_e/(V + V_e)$ is identical with R_F as defined by Consden, Gordon & Martin (1944).

RESULTS

Chromatography of cytochrome c

A preliminary experiment showed that the cytochrome *c* preparation was not chromatographically homogeneous. A homogeneous fraction was obtained by adsorbing the preparation on a column of IRC-50 and eluting with 0.2M sodium phosphate buffer of pH 7.0 and Na^+ concentration, 0.34 g. ions/l. This fraction contained 75% of the material put on the column as measured photometrically at 550 m μ , but only 55% as measured by the ninhydrin reaction. Its spectrophotometric curve was identical with that for oxidized cytochrome *c*. As impurities in the crude cytochrome preparation we observed a pale yellow substance which was eluted ahead of the cytochrome band and a red band which remained stationary at the top of the column. The main band of oxidized cytochrome *c* was just preceded by a band of reduced cytochrome *c*. The main fraction behaved as a single substance when applied to a fresh column of the same resin and could be eluted from it quantitatively (Fig. 1). Cytochrome *c* used in subsequent experiments was purified in this manner.

Paleus & Neilands (1950) and Margoliash (1954*a, b*) have purified cytochrome *c* by chromatography on columns of IRC-50. Margoliash obtained fractions similar to those described here. He examined these spectrophotometrically, determined their iron contents and carried out activity measurements. The pale yellow substance consisted mainly of globin from myoglobin, and the pigment

Fig. 1. Chromatogram of purified cytochrome *c*. Column of IRC-50 17.4 $\times$ 0.9 cm. Buffer: 0.2M sodium phosphate buffer, pH 7.04 and Na^+ concentration 0.34 g. ions/l. Amount on column: 2.4 mg. Temperature: 25°.

adsorbed at the top of the column was oxidized cytochrome *c* of low enzymic activity.

Effects of pH and cation concentration. Figs. 2 and 3 show the effects of pH and cation concentration on the elution volume of cytochrome *c*. The effect of pH is discussed in detail later. The effect of cation concentration was studied at a constant pH of 7.0. The elution volume increased sharply as the cation concentration was reduced below 0.28 g.

Fig. 2. Effect of pH on the elution of cytochrome *c* by sodium phosphate buffers. 6.0 mg. samples chromatographed on columns of IRC-50, 16.0 $\times$ 0.9 cm. Temperature, 25°. Na^+ concentration, ● 0.25 g. ions/l., ▲ 0.34. The broken lines show the amount of Na^+ taken up by the resin: A, from 1.0M NaCl; B, from 0.1M NaCl.

Fig. 3. Effect of cation concentration on the elution of cytochrome *c* at pH 7.0. 6.0 mg. samples chromatographed on columns 16.0 $\times$ 0.9 cm. Temperature, 25°. Buffers: ●, sodium phosphate; ▲, ammonium phosphate; □, sodium phosphate-sodium sulphate.

ions/l., and below 0.22 g. ions/l., the cytochrome band remained stationary at the top of the column. As the cation concentration was increased beyond 0.28, the elution volume gradually decreased to zero. The elution volume was not appreciably altered by substituting NH_4^+ for Na^+ , or Cl^- or SO_4^{2-} for phosphate ions.

Effect of variation in the resin. The elution volume of cytochrome *c* varied considerably with different batches of resin (Table 1). Resin *B* was from the same batch as resin *A*, but of smaller particle size, and it presumably had a greater surface area/g. Since the proteins are too large to penetrate a highly cross-linked resin, interaction between protein and resin takes place on the resin surface, and it might be expected that the elution volume would depend on the surface area/g. of resin and on the surface structure. The success of IRC-50 as a resin for separating and purifying proteins is partly due, no doubt, to the large surface area presented by its sintered structure.

Table 1. *Effect of variation of the resin on the chromatography of cytochrome c*

Eluting buffer: 0.2M sodium phosphate, pH 7.0, $[\text{Na}^+] = 0.34$. Columns contained 2.5 g. dry resin.

Resin	Total volume of effluent (ml.)	Elution volume ($V-V_0$) (ml.)
<i>A</i>	16.3	10.6
<i>B</i>	19.8	13.1
<i>C</i>	15.1	9.4
<i>D</i>	23.7	18.0
<i>E</i>	28.1	—

Fig. 4. Effect of pH on the adsorption of lysine by IRC-50.

Resin *C* behaved not very differently from *A*, but *D* differed considerably from *A* and *C*. Not only was the elution volume greater for *D*, but the protein peak was much broader and the recovery of protein low. The behaviour of resin *E* (Zeo-Carb 226), was very different from that of the samples of IRC-50. The band of cytochrome spread over a considerable part of the column, the resulting peak was very broad and the recovery of protein low. The explanation probably lies in the different surface character of this resin. Resins *A*, *B* and *C* were suitable for the chromatography of proteins.

Adsorption of lysine. Fig. 4 shows the effect of pH on the adsorption of lysine by IRC-50. The amounts of lysine adsorbed/g. of resin have been divided by the corresponding equilibrium concentrations of lysine in the ambient phase, since the resulting ratios are proportional to the distribution coefficients of lysine between the resin and ambient phases. In column experiments, the elution volume of a substance is proportional to its distribution coefficient between the stationary and mobile phases (Consden *et al.* 1944). The behaviour of lysine in the adsorption experiments is compared later with that of cytochrome *c* on columns of IRC-50.

Separation of the haemoglobins

Separation of sheep foetal and bovine CO haemoglobins at 25°. Fig. 5a shows the chromatogram obtained from a mixture of sheep foetal and bovine CO haemoglobins in citrate buffer of pH 5.81 at 25°, using a column 5.0 × 0.9 cm. It was observed that as the haemoglobin bands progressed down the column they left behind a light deposit of coloured material which appeared to be uniformly spread over the whole column. The CO haemoglobins were nevertheless eluted as symmetrical peaks without tailing. At first it was thought that this material remaining on the column was probably denatured haemoglobin (or represented some stage in the denaturation of the protein). It was found that this adsorbed protein could be eluted with concentrated sodium citrate buffer of pH 6.5 and Na^+ concentration 3.2, and peak *C* is the elution peak formed by this material. By running separate chromatograms for sheep foetal and bovine CO haemoglobins it was shown that peak *A* (Fig. 5a) consisted of sheep foetal CO haemoglobin and peak *B* of bovine CO haemoglobin (Boardman & Partridge, 1953). Secondary peaks corresponding to peak *C* were obtained in both cases.

Material from peaks *A* and *B* was rechromatographed, but the same secondary peak was again obtained. This clearly showed that some change in the original material had occurred by passage through the column. If this change were due to denaturation, then yields should be improved by working at lower temperatures.

Fig. 5. Chromatograms of a mixture of sheep foetal CO haemoglobin and bovine CO haemoglobin on columns of IRC-50, columns measuring 5.0×0.9 cm. Buffers: sodium citrate, pH 5.81, Na^+ concentration 0.34 g. ions/l.—changed to sodium citrate, pH 6.5, Na^+ concentration 3.2, after 1.2 ml. (a) Temperature 25° . Amount on column: 2.7 mg. sheep foetal COHb and 3.0 mg. of bovine COHb. (b) Temperature 2° . Amount on column: 2.4 mg. sheep foetal COHb and 4.8 mg. bovine COHb.

Yields of the pure components were increased, in fact, by working at 2° and the results obtained at this temperature are shown in Fig. 5b. Peaks A and B contain 84 % of the original material, compared with 50 % in the experiment at 25° .

Separation of bovine CO haemoglobin and bovine methaemoglobin at 2° . Material from peak C (Fig. 5a) was transferred to a collodion sac and dialysed under pressure. Rechromatography of this material on a fresh column gave only a single peak in the same position as in the original chromatogram. It was also observed that this material was brown in colour and it gave an absorption spectrum similar to that of methaemoglobin. Therefore, a mixture of freshly prepared bovine CO haemoglobin and bovine methaemoglobin was chromatographed with citrate buffer of pH 5.81 and Na^+ concentration, 0.34. No change of buffer was

Fig. 6. Chromatogram of a mixture of bovine CO haemoglobin and bovine methaemoglobin at 2° on a column of IRC-50, column measuring 10.3×0.9 cm. Buffer: sodium citrate pH 5.92, Na^+ concentration 0.34 g. ions/l. Amount on column: 2.6 mg. COHb and 1.7 mg. MetHb.

made in this experiment or in any of the subsequent ones. A complete separation was obtained with CO haemoglobin eluted first as a symmetrical peak and the methaemoglobin eluted later as a very broad peak ($R_f = 0.2$ approximately). The yield of methaemoglobin was low, showing that it was adsorbed to an appreciable extent by the resin at a pH of 5.81.

A much better yield of methaemoglobin was obtained when the mixture of CO haemoglobin and methaemoglobin was chromatographed in citrate buffer of pH 5.92 and Na^+ concentration 0.34 (Fig. 6). Measurement of the absorption spectra showed that the first peak contained CO haemoglobin (yield almost 100 %) and the second contained methaemoglobin (yield 70 %).

It thus appears that the poor yield of pure CO haemoglobins obtained in the original experiments at 25° were due, to a large extent, to the formation of methaemoglobin. As shown in the following experiments, quantitative yields can be obtained by working at the lower temperature (2°), by using freshly prepared proteins and by taking care to prevent methaemoglobin formation by saturation of the buffer with carbon monoxide.

Separation of freshly prepared sheep foetal and bovine CO haemoglobins at 2° . Fig. 7 shows the chromatogram of a mixture of freshly prepared sheep foetal and bovine CO haemoglobins. The buffer was saturated with carbon monoxide. An almost complete separation was obtained with an overall yield of 98 %; approximately 99 % of bovine CO haemoglobin and 97 % of sheep foetal CO haemoglobin.

Separation of sheep foetal and sheep maternal CO haemoglobins at 2° . The result of chromatographing

a mixture of sheep foetal and sheep maternal CO haemoglobins is shown in Fig. 8. In order to resolve these two haemoglobins, it was necessary to use a column 16.2 cm. in length, compared with 8.2 cm. for the separation of sheep foetal and bovine CO haemoglobins. The overall yield was 88 %.

Tests carried out on the CO haemoglobin eluted from the column. Tests carried out on bovine CO haemoglobin which had been chromatographed on a column of IRC-50 at pH 5.81 seem to indicate that the protein was unaltered by passage through the column. Bovine CO haemoglobin is more likely to

be denatured on the column than sheep haemoglobin because of its higher adsorption.

Molecular weight. Osmotic pressure measurements were carried out by Dr G. S. Adair. The results are shown in Table 2. The molecular weight of CO haemoglobin eluted from the column agrees to within 2% with the average value for fresh preparations of bovine CO haemoglobin.

Absorption spectra. The CO haemoglobin eluted from the column, its methaemoglobin and haemoglobin derivatives were examined spectrometrically. All spectrophotometric curves agreed well with those reported in the literature and there was no evidence for the formation of haemochromogen (Lemberg & Legge, 1949).

Fig. 7. Chromatogram of a mixture of freshly prepared sheep foetal CO haemoglobin and bovine CO haemoglobin at 2° on a column of IRC-50, column measuring 8.4 × 0.9 cm. Buffer: sodium citrate pH 5.81, Na⁺ concentration 0.34 g. ions/l. Amount on column: 2.37 mg. sheep foetal COHb and 2.22 mg. bovine COHb.

Fig. 8. Chromatogram of a mixture of sheep foetal CO haemoglobin and sheep maternal CO haemoglobin at 2° on a column of IRC-50, column measuring 16.2 × 0.9 cm. Buffer: sodium citrate pH 5.81, Na⁺ concentration 0.34 g. ions/l. Amount on column: 2.8 mg. sheep foetal COHb and 3.0 mg. of sheep maternal COHb.

Table 2. *Osmotic pressures of bovine CO haemoglobin*

Measured by Dr G. S. Adair. Dialysate: 0.03 M-Na₂HPO₄, 0.01 M-NaH₂PO₄, pH 7.42 at 0°.

	Bovine COHb from column		Bovine COHb (control)	
c (g. protein/100 ml. solution)	1.517	1.472	3.863	1.539
π (osmotic pressure in mm. Hg reduced to 0°)	4.181	4.021	11.59	4.249
$\frac{\pi}{c}$	61 800	62 375	56 600	61 700
$\bar{M}$ (mol.wt.)	64 600	65 131	63 600	64 500
Average $\bar{M}$	—	64 900	—	64 100

Other determinations of the mol.wt. of bovine CO haemoglobin (different preparations) gave 65700 ± 1000 (mean of six determinations) $\pi = \phi \left(10RT \frac{c}{\bar{M}} \right)$; $\phi = \frac{1}{1 - 0.01 b c}$; $b = 2.853 - 0.02109 c$.

Crystallization. CO haemoglobin from the column crystallized more readily than a fresh ordinary preparation. The crystals consisted mainly of long thin needles (Fig. 9a). Fig. 9b shows crystals of bovine CO haemoglobin (prepared as described previously) and Fig. 9c shows crystals of once-recrystallized bovine CO haemoglobin.

DISCUSSION

Chromatography of cytochrome c

The shape of the curve obtained by plotting the elution volume against the cation concentration curve at pH 7.0 may be partly explained on the assumption of a purely electrostatic interaction between protein and resin. Consider the equilibrium of sodium ions, Na^+ , and poly-ions, P^{n+} , between the resin and ambient phases. The exchange may be represented by the equation:

where Z is the number of sodium ions displaced when one poly-ion is adsorbed.

From considerations of mass action (Kunin & Myers, 1950):

$$\frac{[\text{P}_R]}{[\text{P}_S]} \cdot \frac{[\text{Na}_S]^Z}{[\text{Na}_R]^Z} = K,$$

$[\text{P}_R]$ is the activity of poly-ions in the resin phase, $[\text{P}_S]$ is the activity of poly-ions in the ambient phase, $[\text{Na}_R]$ is the activity of Na^+ in the resin phase, $[\text{Na}_S]$ is the activity of Na^+ in the ambient phase; $\frac{[\text{P}_R]}{[\text{P}_S]}$ is the distribution coefficient of the poly-ions between the two phases and is equal to $K_1 V$, where V is the elution volume and K_1 is a constant (Consden *et al.* 1944). Therefore

$$V = K_2 \frac{[\text{Na}_R]^Z}{[\text{Na}_S]^Z}.$$

If $[\text{P}_S]$ is small compared with $[\text{Na}_S]$, $[\text{P}_R]$ will be small compared with $[\text{Na}_R]$, and we may assume that $[\text{Na}_R]$ is virtually unchanged by the adsorption of poly-ions.

As an approximation, we may assume that the Na^+ activity in the resin phase is proportional to the amount of Na^+ taken up by the resin. This varies with the Na^+ concentration in the ambient phase (Hale & Reichenberg, 1949):

$$V = K_3 \left[\frac{\text{Na}^+ \text{ adsorbed by resin}}{[\text{Na}_S]} \right]^Z.$$

Values of V were calculated for varying $[\text{Na}_S]$, putting Z in turn equal to 1, 2, 3, 4, 5 and 6. Fig. 10

Fig. 9. (a) Crystals of bovine CO haemoglobin which had been eluted from a column of IRC-50. (b) Crystals of a fresh preparation of bovine CO haemoglobin. (c) Crystals of once recrystallized bovine CO haemoglobin. Magnification $\times 120$.

Fig. 10. Relationship between the elution volume of a poly-ion and the Na^+ concentration in the ambient phase, derived by a consideration of mass action. —, Theoretical curves; ---, experimental curve for cytochrome c. Z is the number of sodium ions displaced when one poly-ion is adsorbed.

shows the relationship between V and Na^+ concentration in the ambient phase, relative to a fixed value of V at a Na^+ concentration of 0.5. This has been arbitrarily placed at unity. It can be seen that the elution volume versus Na^+ concentration curves rise more steeply as Z increases. The experimental points for cytochrome are also included.

No attempt has been made to fit a theoretical curve to that of cytochrome *c*, since the charge on the protein varies with the ionic strength, and, at the lower Na^+ concentrations, quite a large contribution can be expected from secondary short-range forces. Nevertheless, the above treatment gives a qualitative explanation of the rapid change in elution volume that occurs with increasing Na^+ concentration, in terms of multipoint attachment of the protein molecule to the resin by coulombic forces; the experimental curve arises no doubt by a summation of the effects of electrostatic interaction and secondary forces of shorter range.

The behaviour of cytochrome on the resin column, as shown by the continuous line in Fig. 2 is markedly different from that of an ampholyte of low molecular weight, such as the basic amino acid lysine. Lysine shows a marked maximum adsorption in the region of pH 7. As the pH rises towards its isoelectric point (pH 9.7), the lysine is desorbed and similarly, with falling pH, desorption also takes place due to suppression of the ionization of the resin carboxyl group. Cytochrome *c* (isoelectric point 10.1) is desorbed in a similar manner to lysine as the pH rises from 8 to 10, but it is not desorbed under acidic conditions. On the contrary, at pH 6–6.5 its elution volume rises rapidly and, under still more acidic conditions, the cytochrome band remains stationary at the top of the column. This high adsorption at pH values below 5.8 is not a unique property of cytochrome. A similar behaviour is found with lysozyme and the neutral proteins.

The broken lines in Fig. 2 show the relationship between the amount of Na^+ ion taken up by the resin and the pH (Hale & Reichenberg, 1949; Topp & Pepper, 1949). It will be observed that the rapid rise in the elution volume of cytochrome in sodium buffers occurs over the same pH range as the rapid fall in the amount of Na^+ retained by the resin from buffers of comparable concentration. This suggests that the adsorption of cytochrome *c* that occurs at low pH is due to uncharged carboxyl groups on the resin, and that the mechanism of desorption at higher pH is concerned with the conversion of the resin into its sodium form. Thus, although electrostatic forces diminish as the pH decreases owing to suppression of negative charges on the resin, these forces are replaced by a large increase in the contribution of secondary short-range forces. The increase in short-range forces is most probably due to the concomitant removal of a layer of electro-

statically bound water. With substances of high molecular weight, such as the proteins, the contribution of short-range forces towards the net adsorption affinity may be very large and may be sufficient to account for the high adsorption at low pH. With amino acids this contribution will be small and thus the observed desorption of lysine at low pH would be expected. In general the secondary forces may be regarded as hydrogen bonds and van der Waal forces, but it would appear from the chemical structure of the resin that hydrogen bonds play the major role. The resin has a very large number of carboxyl groups close together, but no large non-polar groups.

The electrostatic attraction between protein and resin will depend on the net charge on the protein molecule and, to a lesser extent, on the distribution of that charge. The secondary forces will be influenced by the configuration of the protein molecule, the nature of the side chains and the amount of water bound by the protein. It should thus be possible to separate proteins of the same net charge but with different molecular configurations, or proteins of the same molecular weight but with different net charges. Lysozyme and cytochrome *c* have approximately the same isoelectric point (10.5, 10.1) and molecular weight (15 000, 13 000), but their elution volumes are very different. With a column of IRC-50, 5 cm. $\times$ 0.9 cm., cytochrome is eluted with 0.2M sodium phosphate buffer, pH 7.0, in 3.3 ml. and lysozyme in 26 ml. An example of the second type of separation is that of the carboxy-haemoglobins.

Separation of the haemoglobins presents two major difficulties. First, the proteins are readily denatured and at their isoelectric points slowly form precipitates even at 0°. Secondly, the proteins are isoelectric near neutrality and thus the flat section of the adsorption/pH curve between pH 7 and 9, found with cytochrome *c* is absent. The isoelectric points of bovine, sheep maternal and sheep foetal CO haemoglobins are respectively 7.27, 6.90 and 6.66, at 0° in phosphate buffer of ionic strength 0.02 (Adair, private communication), but in the 0.1M citrate buffers used for the chromatographic experiments their isoelectric points lie somewhere between 4.5 and 5.6. The CO haemoglobins were individually subjected to electrophoresis in the apparatus of Tiselius (1937) and in 0.1M citrate buffer of pH 5.81 and Na^+ ion concentration 0.34 each moved towards the positive pole. Thus in citrate buffer at pH 6, all the CO haemoglobins have a net negative charge and none is adsorbed by the resin. On the other hand, as is shown in Fig. 2, at pH 5 the carboxyl groups of the resin are almost wholly undissociated and adsorption of the haemoglobins is complete. The elution volume/pH data of the haemoglobins may thus be represented by a

family of steep curves falling rapidly from very high values to zero in the narrow range pH 5–6, the curves for the more acidic proteins being displaced to the left of those for proteins with higher isoelectric points. Owing to the extreme steepness of the curves, chromatographic behaviour is sharply dependent upon the pH and cation concentration of the buffers, and these must be accurately controlled. In the experiments described here, the pH was controlled to ± 0.02 .

Closely related neutral proteins, such as the haemoglobins, may be successfully separated by elution chromatography using columns of IRC-50. The separation of proteins by chromatography is complicated by denaturation of many proteins on most of the common adsorbents. Denaturation or partial denaturation of a protein such as CO haemoglobin may take place if the protein is adsorbed very strongly on IRC-50. Thus, for the successful separation of neutral proteins which are susceptible to denaturation, it is best to arrange conditions so that all the proteins in the mixture move down the column with R_F values > 0.5 . This can be done by a suitable selection of the pH and Na^+ concentration of the eluting buffer. If more than one protein of the mixture move with an $R_F = 1$, then the fraction containing these proteins should be rechromatographed under slightly more acidic conditions.

SUMMARY

1. The effect of pH and cation concentration of buffer on the adsorption of cytochrome *c* on the ion-exchange resin, IRC-50, has been studied in some detail. The behaviour of the protein on the resin is markedly different from that of ampholytes of low molecular weight such as the basic amino acids. Lysine is desorbed as the pH falls below 5 owing to suppression of the ionization of the resin carboxyl groups, but the adsorption of the protein is greatly increased under acidic conditions. It is suggested that this adsorption is due to a large increase in secondary short-range forces.

2. Some closely related neutral proteins have been separated by elution chromatography on columns of IRC-50. Sheep foetal carbon monoxide haemoglobin has been separated from sheep maternal CO haemoglobin and from bovine CO haemoglobin and bovine CO haemoglobin from bovine methaemoglobin. The separation of the

haemoglobins is sharply dependent on the pH and sodium ion concentration of the eluting buffer. To obtain good yields, it is necessary to work at a temperature near 0° and to use freshly prepared proteins.

3. Tests carried out on bovine CO haemoglobin eluted from the column indicate that the protein was unaltered by passage through the column. The material from the column could be readily crystallized.

The authors wish to thank Dr G. S. Adair, F.R.S., for helpful discussions during the course of the work. Thanks are also due to Dr Adair for determining the molecular weight of the eluted CO haemoglobin and for help in the recrystallization experiments. The authors are indebted to Dr R. S. Comline for gifts of sheep foetal and sheep maternal blood. Part of this work was carried out during the tenure by one of us (N.K.B.) of a Dafydd Lewis Trust (Melbourne) Scholarship.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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