REFERENCES

- Association of Official Agricultural Chemists (1947). *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, 2nd ed. Washington.
- Aubel, E. (1938). *C.R. Soc. Biol., Paris*, **128**, 45.
- Chibnall, A. C., Rees, H. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
- Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
- Elsden, S. R. (1945). *J. exp. Biol.* **22**, 51.
- Elsden, S. R. & Sijpesteijn, A. K. (1950a). *J. gen. Microbiol.* **4**, xi.
- Elsden, S. R. & Sijpesteijn, A. K. (1950b). Personal communication.
- Johns, A. T. (1949). *Nature, Lond.*, **164**, 620.
- Lewis, D. (1951). *Biochem. J.* **48**, 175.
- Lugg, J. W. H. (1938). *J. agric. Sci.* **28**, 688.
- Phillipson, A. T. (1942). *J. exp. biol.* **19**, 186.
- Phillipson, A. T. & Innes, J. R. M. (1939). *Quart. J. exp. Physiol.* **29**, 333.
- Quastel, J. H. & Stephenson, M. (1925). *Biochem. J.* **19**, 660.
- Quastel, J. H., Stephenson, M. & Whetham, M. D. (1925). *Biochem. J.* **19**, 304.
- Sapiro, M. L., Hoffund, S., Clark, R. & Quin, J. I. (1949). *Onderstepoort J. vet. Sci.* **22**, 357.
- Sijpesteijn, A. K. (1949). *Antonie van Leeuwenhoek*, **15**, 49.
- Sijpesteijn, A. K. (1950). Private communication.
- Stephenson, M. & Stickland, L. H. (1931). *Biochem. J.* **25**, 205.
- Stickland, L. H. (1931). *Biochem. J.* **25**, 1543.
- Woods, D. D. (1938). *Biochem. J.* **32**, 2000.

Displacement Chromatography on Synthetic Ion-exchange Resins

7. SEPARATIONS USING A STRONGLY BASIC RESIN

BY S. M. PARTRIDGE AND R. C. BRIMLEY

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

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Previous communications (Partridge, 1949, 1950) have reported the use of an ion-exchange resin for the separation of amino-acids from a protein hydrolysate into eight groups, each consisting of no more than three amino-acids. The separation was carried out as a displacement chromatogram, using a strongly acidic cation-exchange resin, and displacing the mixture with ammonia or sodium hydroxide solution. A fractionation of this kind forms a logical starting point for a scheme aiming at the complete separation and isolation of all the component amino-acids in the complex mixture; and the preliminary group separation has thus been termed the 'primary fractionation'. When carried out with a multiple column having a top section packed with sulphonated polystyrene resin (Partridge, Brimley & Pepper, 1950) the primary fractionation results in the immediate isolation of three amino-acids in a pure condition; these are aspartic acid, lysine and arginine. The other amino-acids appear as mixed bands as follows: band II, glutamic acid, serine and threonine; band III, glycine and alanine; band IV, valine and proline; band V, methionine, leucine and isoleucine; band VI, histidine and the leucines.

Various procedures have been described for the further separation of the components of some of these bands, e.g. the separation of glutamic acid from band II by the use of a weakly basic resin

(Partridge & Brimley, 1949) or the separation of methionine from leucine by the use of a cation-exchange resin at higher temperatures (Partridge & Brimley, 1951). However, the appearance in commercial production of several new strongly basic resins now offers the possibility of separating other mixed bands and also of bringing all the secondary separations within a generalized scheme.

The separation of a series of amino-acids in displacement chromatograms using strongly acidic resins is determined largely by the order of the values of pK_1 (COOH) for the members of the series (Davies, 1949). Similarly, the order of displacement from strongly basic anion-exchangers is largely determined by the order of pK_2 (NH_3^+) (Davies, Hughes & Partridge, 1950) and thus displacement from an anion-exchanger should give rise, not to a simple reversal of the order found with cation-exchangers, but rather to an entirely new grouping of the amino-acids. The work described in this paper shows that this prediction is borne out in practice and that use may be made of the differences for securing further separations.

EXPERIMENTS AND RESULTS

The ion exchanger used for these experiments was Dowex 2 (obtainable from the Dow Chemical Company, Midland, Michigan). The resin is a cross-

linked polymer containing strongly basic quaternary groups. It is a cream-coloured material, and the sample used was in the form of micro-beads 250–500 mesh/in. The resin was supplied in the chloride form, and was regenerated before use with carbonate-free 2N-sodium hydroxide solution until chloride-free. (It was found convenient to prepare carbon dioxide-free alkali by adding 10 g. barium hydroxide crystals to each l. of 2N-NaOH solution, the solution being filtered immediately before use). After regeneration the resin was washed with carbonate-free distilled water until the alkalinity of the eluate fell below pH 8. Carbonate-free solutions were used at all stages, since it was found that if any carbonate became adsorbed on the resin, carbon dioxide was released by the acid used as displacement developer, and this seriously disturbed the chromatogram.

The preliminary characterization of the resin has already been reported (Davies *et al.* 1950), the results showing that the widths of the boundaries (as defined by Partridge & Westall, 1949) for hydrochloric acid were 1–1.5 cm. when the rate of progression of the boundaries was in the range 2–4 cm./hr. The boundaries were considerably wider than those observed with the cation exchanger Zeo-Karb 215 at comparable rates of flow, and for this reason, in separation experiments with Dowex 2, the flow rate was adjusted to maintain a rate of progression of the boundaries below 3 cm./hr. The resin behaved unfunctionally for strong acids, its capacity being 3.7 m-equiv./g. dry resin.

Construction of the column

The column was prepared in two sections connected in series and mounted one above the other. The upper section consisted of a filtration tube of diameter 1.6 cm. and height 15 cm. It was packed with 10 g. (air-dry wt.) of Dowex 2 (water content, 16.0%), the height occupied by the resin being 11.5 cm. The lower filtration tube was 1.0 cm. in diameter and 11 cm. in height. It contained 3 g. of resin of the same particle size as that used for the upper section (height occupied by the resin, 8.4 cm.). The general arrangement of the assembly was that described by Partridge & Brimley (1951). Before use the column was treated alternately with 0.2N-HCl and CO₂-free 2N-NaOH in order to remove impurities and compact the resin bed. The column was finally regenerated and washed as described above.

Separation experiments

The plan adopted for these experiments was to carry out a standard displacement procedure with a series of known amino-acid mixtures representing the mixed bands obtained in the primary fractionation. The amount of amino-acid mixture was approximately 12 mmol. in each case. The mixture was dissolved in water (70 ml.) and boiled to expel carbon dioxide. After cooling the solution was applied to the column at a rate of 40–50 ml./hr. and

the column then washed with a little carbon dioxide-free water. The displacement developer (0.1N-hydrochloric acid, 650 ml.) was then applied at the same rate. The effluent was collected in fractions by an automatic device which changed the receiver at pre-set intervals of time. The column was usually allowed to run unattended overnight, and the following morning one drop was taken from each receiver for the preparation of a filter-paper chromatogram. The results of the qualitative analyses so obtained are shown in Fig. 1.

Fig. 1. Chromatograms prepared by displacing mixtures of (a) alanine and glycine, (b) proline and valine, (c) serine and threonine, (d) leucine and methionine from a column packed with Dowex 2. Displacement developer, 0.1N-HCl.

Fig. 1 (a), (b) and (d) shows that excellent separations were obtained with mixtures of alanine and glycine, proline and valine, and leucine and methionine respectively; however, serine and threonine showed no tendency to separate.

The isolation of L-alanine and glycine from the hydrolysis products of egg albumin

The primary fractionation of an acid-hydrolysate of commercial egg albumin has already been described (Partridge, 1949). Since, at the time this fractionation was carried out, no satisfactory procedure was available for the separation of glycine from alanine, the two amino-acids (comprising band III) had been collected as a crystalline mixture for storage. This mixture was used for the present experiments.

The column used in the fractionation experiments described above was adapted for a larger output by introducing a third and larger section. This was mounted on top of the existing assembly and was 2.1 cm. in diameter and 22 cm.

in height. It was packed with Dowex 2 in bead form, wet screen analysis 100–200 mesh/in., the resin occupying a height of 17.7 cm. in the filtration tube. The mixture of glycine and alanine (4.0 g.) was dissolved in water (100 ml.), brought to the boil to expel CO_2 and cooled. It was applied to the column at a rate of 30 ml./hr. and was followed by the displacement developer (0.15N-HCl) which was applied at the same rate. The fraction collector was set to change the receiver every 20 min. After completion of the experiment a qualitative analysis of the contents of each receiver was carried out by means of paper chromatography. The first twenty-one fractions contained alanine only. This was followed by two fractions which contained a mixture of glycine and alanine, and which were rejected. The following ten fractions contained glycine only, while the 34th fraction also contained glycine, but was contaminated with HCl and was rejected.

Recovery of L-alanine. The solution containing L-alanine was concentrated under reduced pressure to the point at which crystallization began. Two volumes of hot ethanol were then added to the warm solution. On cooling, L-alanine crystallized in fine needles, wt. 2.54 g. Found: N 15.7. Calc. for $\text{C}_3\text{H}_7\text{O}_2\text{N}$: N, 15.7%; $[\alpha]_D^{20} + 14.5^\circ$ in 5.9N-HCl (c, 3.37, 2 dm.). The yield represented 3.44% of the dry weight of protein taken.

Recovery of glycine. The glycine solution was concentrated under reduced pressure to a colourless viscous liquid. About one-half the volume of ethanol was then added, and on scratching the glycine crystallized in small prisms, wt. 1.07 g. Found: N, 18.4. Calc. for $\text{C}_2\text{H}_5\text{O}_2\text{N}$: N, 18.7%. The yield represented 1.44% of the dry weight of protein taken.

DISCUSSION

The mechanisms which control the separation of a mixture of amino-acids in a displacement chromatogram have been discussed by Davies (1949). Considering the case of displacement from a column of strongly acidic resin, Davies pointed out that separation would be expected to depend upon: (a) the extent of ionization of the individual amino-acids in their passage down the column, (b) the charges carried by the ions present, and (c) the van der Waals forces between the resin and the various species present. It was further shown that, for a cation-exchange column, mechanism (a) would result in an order of displacement for a series of amino-acids which would be qualitatively identical with the order of dissociation constants for the process $\text{A}^+ \rightleftharpoons \text{H}^+ + \text{A}^\pm$; that is to say, the order of $\text{pK}_1(\text{COOH})$ for monoamino-acids. The operation of mechanism (b) would be expected to result in a retardation of the diamino-acids since they are able to form bivalent cations. Similarly, the operation of mechanism (c) should result in a greater relative retardation of those amino-acids of higher molecular weight, or those possessing a specific structure such as the aromatic nucleus which would confer a higher adsorptive affinity towards the resin.

In the case of a displacement chromatogram carried out with a strongly basic anion-exchanger,

mechanism (a) becomes dependent upon the dissociation constant for the reaction $\text{A}^\pm \rightleftharpoons \text{H}^+ + \text{A}^-$; that is to say, upon the values of $\text{pK}_2(\text{NH}_3^+)$ for monocarboxylic amino-acids; and in this case amino-acids forming bivalent anions should be strongly retarded (mechanism b). The effect of van der Waals forces should operate in the same sense in both cases; amino-acids associated with a large van der Waals contribution being retarded with both types of resin.

The recent introduction of resins with strongly basic properties has enabled this hypothesis to be tested by a direct comparison of the order of displacement of the same set of amino-acids with resins of both types. The order of displacement of a series of amino-acids from Dowex 2 was determined by direct experiment. A mixture of fourteen amino-acids was displaced from a column with 0.1N-hydrochloric acid and the effluent from the column analysed by paper chromatography. The result is given in Table 1 (column 4) which includes, for comparison, values of $\text{pK}_2(\text{NH}_3^+)$ taken from Cohn & Edsall (1943). Arginine was only slightly retarded by the resin, and appeared as an 'elution peak' in front of the main displacement pattern. The other amino-acids followed in approximately the expected order, but proline, histidine, methionine and phenylalanine appeared rather lower in the order than would be expected from their dissociation constants. The amino-acids included in the brackets formed mixed bands, and as will be seen from the table, three amino-acids, valine, leucine and glycine, which form a mixed band, have almost identical values of pK_2 .

Table 1 (column 1) also includes the order of displacement (Partridge, 1949) of the amino-acids from Zeo-Karb 215, a strongly acidic resin. The order is approximately that expected from the values of pK_1 , but here again proline, methionine and phenylalanine are relatively retarded. A high contribution from van der Waals forces would be expected for phenylalanine and to a lesser extent for methionine since these amino-acids are strongly adsorbed by charcoal (Tiselius, 1947). Proline is not strongly adsorbed by charcoal, however, and this may be taken to suggest that a further mechanism leading to retardation may be operative.

Column 2 (Table 1) lists the order of displacement of the amino-acids from Zeo-Karb 215 at a higher temperature. It will be seen that the order is somewhat different from that at 20° , and that the behaviour of proline and methionine more nearly approaches that expected from the values of pK_1 . This indicates that the effect of the van der Waals contribution for these amino-acids is relatively less important at 60° than at 20° . Use has been made of the change in order to secure the isolation of valine, proline, methionine and leucine from the hydrolysis products of a protein (Partridge & Brimley, 1951).

1. The use of a strongly basic anion-exchange resin (Dowex 2) for the separation of amino-acids by displacement chromatography has been investigated.

2. Mixtures of the following pairs of amino-acids were separated: glycine-alanine, valine-proline, methionine-leucine. A mixture of serine and threonine did not separate.

3. The isolation of glycine and L-alanine from a protein hydrolysate is described.

4. The order of displacement of fourteen amino-acids was determined by displacing the mixture with dilute hydrochloric acid solution from a column of Dowex 2. This order was substantially the same as the order of pK_a (NH_3^+) for the monocarboxylic

amino-acids, and the result is discussed in relation to the theory of Davies (1949).

5. The use of a strongly basic resin in a simple scheme for the isolation of amino-acids from complex mixtures is discussed.

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REFERENCES

- Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides*. New York: Reinhold.
- Cosgrove, J. D. & Strickland, J. D. H. (1950). *J. chem. Soc.* p. 1845.
- Davies, C. W. (1949). *Biochem. J.* **45**, 38.
- Davies, C. W., Hughes, R. B. & Partridge, S. M. (1950). *J. chem. Soc.* p. 2285.
- Gregor, H. P. (1948). *J. Amer. chem. Soc.* **70**, 1293.
- Partridge, S. M. (1949). *Biochem. J.* **44**, 521.
- Partridge, S. M. (1950). *Chem. & Ind.* **20**, p. 383.
- Partridge, S. M. & Brimley, R. C. (1949). *Biochem. J.* **44**, 513.
- Partridge, S. M. & Brimley, R. C. (1951). *Biochem. J.* **48**, 313.
- Partridge, S. M., Brimley, R. C. & Pepper, K. W. (1950). *Biochem. J.* **46**, 334.
- Partridge, S. M. & Westall, R. G. (1949). *Biochem. J.* **44**, 418.
- Tiselius, A. (1947). *Advanc. protein Chem.* **3**, 67.
- Weiss, D. E. (1950). *Nature, Lond.*, **166**, 66.

The Micro-estimation of Porphyrins by Copper Titration

By I. T. OLIVER AND W. A. RAWLINSON

Department of Biochemistry, University of Melbourne, Australia

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Much of the work dealing with porphyrins requires the handling of small amounts of pigments, usually of the order of micrograms. It is thus rarely possible to isolate a sufficient quantity of material of proved homogeneity suitable for carbon, hydrogen, oxygen or nitrogen analysis. Nevertheless, the highly characteristic absorption spectra of porphyrin pigments have enabled workers to make great progress in the field, as structural modifications are reflected strongly in the nature of the spectra.

In the present paper a problem of current interest concerning the porphyrin *a* isolated from the prosthetic group of cytochrome *a* of ox-heart muscle (Rawlinson & Hale, 1949) is taken as an illustration of the use of spectrophotometry in making accurate estimates of pigment concentrations.

The immediate aim was to determine molecular concentrations of porphyrin *a* for the calculation of molar extinction coefficients. The method described here utilizes the ability of free porphyrins to form metalloporphyrins which possess quite different light-absorbing properties. The conditions found most suitable involved the titration of free porphyrin

in glacial acetic acid with a standard solution of cupric acetate in the same solvent. The formation of copper complex is quantitative, and no degradation of pigment is evident during the short period of heating necessary for the reaction. Although the disappearance of the red fluorescence of the free porphyrin, under ultraviolet radiation, can be used to give an approximate measure of the completion of the reaction, the end point is determined with much greater accuracy by measurements of the change of optical density at any wavelength where the two components have marked absorption differences. The reaction involves the replacement of the two hydrogen atoms bound to the nitrogen of each free porphyrin molecule by one cupric ion, so that the least number of metal ions required to produce the maximum change of optical density is equivalent to the number of free porphyrin molecules originally present.

The principles outlined might prove of use for the estimation of other pigments of unknown constitution, where quantitative conversion to another derivative of sufficiently different absorption spectrum is possible.