Displacement Chromatography on Synthetic Ion-exchange Resins

2. THE SEPARATION OF ORGANIC ACIDS AND ACIDIC AMINO-ACIDS BY THE USE OF ANION-EXCHANGE RESINS

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The application of the principle of displacement chromatography to the separation of bases and amino-acids on columns packed with the cation-exchange resin Zeo-Karb 215 has been described in Part 1 of this communication (Partridge & Westall, 1949). It is expected that this method will prove to be of value in the isolation of bases and amino-acids from biological extracts of various kinds, and that it will also facilitate the fractionation of protein hydrolysates on a rather larger scale than has been possible hitherto. Since all the amino-acids commonly found in proteins are adsorbed by Zeo-Karb 215 and other cation exchangers of a similar type, the use of these resins constitutes a means of carrying out the first fractionation of a protein hydrolysate into a series of groups of amino-acids of similar affinity for the cation exchanger.

The data given in Part 1 show that although aspartic acid should be separable from a protein hydrolysate in a pure condition, glutamic acid will appear in a mixed fraction containing serine and threonine; thus further means are required to separate glutamic acid from the two hydroxyamino-acids. Since several excellent methods have been described for the separation of glutamic and aspartic acids from each other (Drake, 1947; Consden, Gordon & Martin, 1948) and also from other less acidic amino-acids (Freudenberg, Walch & Molter, 1942; Cleaver, Hardy & Cassidy, 1945; Tiselius, Drake & Hagdahl, 1947) by the use of synthetic resinous anion exchangers, the application of a chromatographic technique based on one of the commercially obtainable anion-exchange resins obviously presents itself. However, the methods cited have been designed, for the most part, for use on a smaller scale than is envisaged here, and for that reason it was considered worth while to reinvestigate a number of anion exchangers by methods conforming to the general pattern of those described in Part 1.

EXPERIMENTAL

Materials

Samples of three commercial resins have been examined. These were: Wofatit M; Amberlite IR4 (Resinous Products

and Chemical Company, Philadelphia); De-Acidite B (Permutit Company, London). We are indebted to the Director of the Chemical Research Laboratory, Teddington, for the gift of the sample of Wofatit M, supplies of which are at present difficult to obtain.

Methods

The apparatus used and the methods adopted for the determination of the adsorption capacity of the resins and the width of the boundaries are generally as described in Part 1. It should, however, be pointed out that the behaviour of the anion exchangers is markedly different from that of the highly acidic base-exchange resins in several important respects. These differences in behaviour are due especially to the fact that exchange reactions taking place on the anion exchangers are much slower than those observed on resins containing $-\text{SO}_3\text{H}$ groups. In addition, commercial resins of the condensed polyamine type contain weakly acidic groups, which give the resin an amphoteric character in neutral or weakly alkaline solutions. This latter property causes considerable difficulty in regenerating the resin, particularly as it is desirable to prepare the resin base in a substantially salt-free condition.

Various methods of regeneration have been used to suit particular experimental conditions, and details of the procedures adopted appear in the relevant sections below.

The determination of titration curves. The experimental procedure was identical for the three resins investigated. The sample of resin was ground in the dry condition in a hammer mill and graded between wire sieves (80–100 mesh/in.). The powder was allowed to stand overnight in 0.1 N-HCl and was washed free from fine particles by means of an upward flow of water. It was then packed into a tall glass filtration tube and treated three times alternately with 0.1 N-HCl and N-NaOH, the final washing from N-NaOH being carried out with a large volume of distilled water until the specific conductivity of the effluent solution was less than 10 gemmhos. The powdered resin was then dried in air at room temperature and a sample taken for the determination of moisture content by drying to constant weight over P_2O_5 *in vacuo* at 80°. Twenty to thirty samples of the air-dried resin, each about 0.6 g., were then weighed into 50 ml. flasks; 20 ml. of 0.5 M-NaCl were added to each flask and the volume made up to 40 ml. by the addition of various amounts of HCl or NaOH. The flasks were then tightly stoppered and allowed to stand with occasional shaking for 48 hr., when the supernatant solution was withdrawn from each flask for titration and for the determination of pH (glass electrode).

RESULTS

The effect of pH on the retention of anions. The anion-exchange resins at present available commercially are rather complex in structure, and usually contain a wide range of reactive groups which vary considerably in their strength as bases. The following functional groups are commonly present in such resins: —NH_2 (aromatic or aliphatic), =NH (aromatic or aliphatic), ≡N (aliphatic) (cf. Myers, 1942; Davies, 1948). In addition, weakly acidic phenolic hydroxyl groups may be present.

The complexity of the ionizing system in such resins may be illustrated by the example of one commercial anion exchanger which is said to be prepared by the co-condensation of ethylenediamine with phenol and formaldehyde, cross links being formed by the reaction of the condensed product with ethylene dichloride. Such a resin would be expected to contain —NH_2 (aliphatic), =NH (aliphatic), ≡N (aliphatic) and —OH (aromatic), and its titration curve would, therefore, show the presence of a whole series of ionizing groups over a considerable pH range.

The titration curves of three commercial resins are given in Fig. 1. The determinations were carried out by the method already given, and it should be observed that the curves represent the adsorption of Cl^- or Na^+ from solutions containing a relatively high concentration (0.25M) of sodium chloride. In the curves, the amount in m-equiv. of Cl^- or Na^+ adsorbed/g. (dry wt.) of resin is plotted against the pH of the supernatant solution. It will be seen that in each case the adsorption of Cl^- increases steadily with decreasing pH from pH 9 to 1, and that at pH 9–10 and above there is a nett adsorption of Na^+ . Thus the resins (in different degrees) have weakly acidic properties in addition to their main character as bases.

With each sample of resin there is a point in the region of pH 9–10 at which the well-washed resin retains neither free acid nor free base and thus behaves as an amphoteric substance at its isoelectric point; but it should be noted that at this point the adsorption capacity of the resin is not necessarily zero, since both cations and anions may be removed from the solution in equal amounts. Indeed in the case of a resin containing phenolic hydroxyl as the base-reacting radical, the adsorption of cations at pH 9–10 may be considerable, and, if the resin has an 'isoelectric point' in this region, it must be expected to retain both anions and cations in significant amounts when equilibrated with weakly alkaline solutions containing salts. This expectation is confirmed by experience, since, if the resins are brought to their free-base condition by buffering to pH 9–10 with borate or carbonate-bicarbonate

mixtures, and are subsequently washed with distilled water, the removal of salt (or free alkali produced by hydrolysis of the salts of weak acids) from the resin proves to be an extremely slow process; considerable quantities of electrolyte are washed from the resin before the conductivity of the effluent solutions approaches that of the distilled water used for washing.

Fig. 1. Titration of three commercial anion-exchange resins in the presence of 0.25M-NaCl: curve A, Amberlite IR 4; curve B, Wofatit M; curve C, De-Acidite B.

This is an important consideration wherever large quantities of resin are to be used, and, in fact, the amount of distilled water required to wash a large column free from electrolytes after it has been regenerated with alkali or alkaline buffers may be prohibitive. For the practical purposes of this work, it has, therefore, been considered preferable to choose a cycle of operations which avoids the necessity of bringing the resin into equilibrium with alkaline solution; and, as will appear later, a further advantage in limiting the adsorption-regeneration cycle to pH 1–7 lies in the fact that, over this range, expansion and contraction of the resin is reduced. However, some of the small-scale laboratory experiments (such as the determination of adsorption isotherms) call for a fully regenerated resin, and these were carried out with resin samples that had been regenerated with dilute sodium hydroxide solution.

Titration curves for a sample of Amberlite IR 4 have recently been given by Kunin & Myers (1947), who carried out the determinations both in the presence and the absence of neutral salt. These curves show clearly that the free-base form of the resin may be brought, by exhaustive washing, into equilibrium with distilled water at a pH approaching 7, although the resin (which contains primary aliphatic amino groups) is a fairly strong base. This is to be expected since the resin is insoluble, and charge effects tend to prevent the release of free hydroxyl ions into the aqueous phase. On addition of a neutral salt, however, the pH of the solution rises, presumably because soluble cations become available, and the supernatant solution acquires a concentration of hydroxyl ions sufficient to bring about an equilibrium of essentially the same kind as that occurring when a soluble base is dissolved in water containing electrolytes.

The width of the boundaries. In Part I an expression was derived by which the width (λ_{90}) of a boundary formed by a solute on a column could be calculated from a knowledge of the shape of the solute front in the effluent:

$$\text{Width of the boundary in cm. } (\lambda_{90}) = \left(\frac{V_{90} - V_0}{V_e} \right) L.$$

The notation used is that given in Part I. The results of a number of such determinations on the cation-exchange resin Zeo-Karb 215 are shown in Part I, Fig. 6 (p. 425), which shows the effect of the particle size of the resin and the rate of progression of the boundary down the column on the width of a boundary due to 0.042N-NH₃.

Fig. 2 shows the widths of boundaries due to 0.0495M-glutamic acid on various anion-exchange resins. The experimental procedure adopted was that described in Part I (p. 425). Curve A (Fig. 2) gives the widths of the boundaries on a column of 9.0 mm. diameter $\times$ 103 mm. height packed with De-Acidite B (80–100 mesh/in.). The De-Acidite B, after treating alternately with N-NaOH and 0.1N-HCl, was regenerated with N-NaOH solution and washed with distilled water to a low conductivity. Curve B was obtained with Amberlite IR 4 (80–100 mesh/in.) which was regenerated by equilibration with 0.0005 N-HCl after treating alternately with 0.1N-HCl and water. Curve C was obtained with Wofatit M (80–100 mesh/in.) which was regenerated by the method used for the sample of De-Acidite. Curve D refers to a column packed with Amberlite IR 4 that had been treated alternately with 0.1N-HCl and water and regenerated with phosphate buffer of pH 7.0 followed by thorough washing with distilled water to low conductivity.

It will be observed that, with the anion-exchange resins, the effect of the rate of flow in the column is very much more marked than is the case with Zeo-

Karb 215, and that the boundaries (with comparable rates of flow) are very much wider in all cases. Indeed it is clear that, with all the samples, the rate of progression of the boundary down the column must be limited to less than 2 cm./hr. in order to obtain separations of the same sharpness as those taking place on Zeo-Karb 215 at a comparable rate of 15 cm./hr.

The adsorption of a weak acid such as glutamic acid on the anion exchangers is, therefore, a very slow process as compared with the replacement of H⁺ by cations on strongly acidic resins such as Zeo-Karb 215, and, since parallel experiments with aspartic acid and lactic acid gave similar results, it

Fig. 2. Width of boundaries formed by glutamic acid (0.05M) on various anion-exchange resins (80–100 mesh/in.): curve A, De-Acidite B; curve B, Amberlite IR 4 (regenerated with 0.0005N-HCl); curve C, Wofatit M; curve D, Amberlite IR 4 (regenerated with phosphate buffer of pH 7).

may be assumed that the slow rate of reaction applies to organic acids as a whole. Since the boundary widths found for 80–100 mesh/in. resin were so large, it was not considered useful to examine the properties of resin samples of larger particle size, and the finer grade of resin was used throughout the remainder of the investigation; in separation experiments the rate of progression of the boundaries down the column was adjusted to 3–4 cm./hr., since slower rates than this were not considered to be practical except under special circumstances.

The data given in Fig. 2 provide a rational basis for the choice of a suitable resin for the purpose of fractionation experiments. Curve D shows that the

boundaries on the column of Amberlite (regenerated to pH 7) are considerably narrower than those given by any of the other resin samples. However, the adsorption capacity of the resin should also be taken into account in considering the performance of a column, since it is clear that although the boundary given by Amberlite IR 4 (regenerated to pH 7) is 2.75 cm. at a rate of progression of 6 cm./hr., and that given by the same resin regenerated with 0.0005N-HCl is 5.75 cm., in the former case the capacity of the resin for anions is about twice that in the latter. Thus for a given quantity of solute the band on the column is approximately one-half the height when the column is regenerated to pH 7, and consequently the proportion of pure solute recoverable should be approximately the same in both cases. However it must be borne in mind that regeneration to pH 7 would allow the use of a shorter column.

For the purpose of comparing the performance of resins, we may therefore compare the values of $\lambda_{90} \times \epsilon$ for a given solute at a given rate of progression of the boundary. This comparison is set out in Table 1 for boundaries given by glutamic acid, and, from these data, it is clear that the performances of all the anion-exchange resins on this basis do not greatly differ.

The suitability of a resin for a given separation must remain a matter to be decided in relation to particular circumstances regarding both the type of separation and the apparatus available. Resins of lower adsorptive capacity suffer the disadvantage that longer columns are required for the same throughput, but in the present work it was considered preferable to use long columns packed with De-Acidite B since the chemical and mechanical stability of this resin made it convenient to handle in the finely powdered condition.

The data for the cation-exchange resin Zeo-Karb 215 are included in Table 1 to serve as a basis for comparison. The superiority of the cation exchanger at high rates of flow is most striking, and is in conformity with our experience in fractionation experiments on the two kinds of resin. Displacement chromatography on commercial anion-exchange resins was successful only if carried out slowly and on long columns packed with finely ground material.

The retention of organic acids on De-Acidite B. Fig. 3 shows a number of 'retention isotherms' which were determined on a sample of De-Acidite B (1.60 g. dry wt.) that had been treated several times alter-

nately with 0.1N-HCl and N-NaOH, and regenerated with N-NaOH solution. The values of ϵ were calculated from the retention volumes (V_e) given by a column 58 mm. in height and 9.0 mm. in diameter. Details of the experimental procedure for the determination of ϵ are given in Part 1, p. 421.

Fig. 3. The retention of a number of acids on De-Acidite B (80–100 mesh/in.). The experiments were carried out with a column 9 mm. in diameter containing 1.6 g. resin.

The adsorption of the neutral amino-acids, serine, glycine and alanine, on De-Acidite B was very small and for practical purposes may be neglected. The two acidic amino-acids, aspartic acid and glutamic acid, were well adsorbed, and, as would be expected from its lower isoelectric point, the value of ϵ (mmol. of solute adsorbed/g. resin dry wt.) for aspartic acid was rather higher than that for glutamic acid. A feature of the group of curves given in Fig. 3 is that the adsorption capacity of the resin for the strong mineral acids is three to four times that for aspartic and glutamic acids. This was to be expected from the titration curve of the resin (Fig. 1) which demonstrates the presence of a range of basic groups of varying strengths. Thus hydrochloric acid may be retained by weakly basic groups in the resin which remain un-ionized in the presence of the acidic amino-acids.

Table 1. Comparison of $\lambda_{90} \times \epsilon$ for glutamic acid boundaries on various resins

Resin	ϵ (mmol./g.)	S (cm./hr.)	$\lambda_{90} \times \epsilon$	S (cm./hr.)	$\lambda_{90} \times \epsilon$
De-Acidite B (fully regenerated)	0.85	5	5.1	10	10.8
Amberlite IR4 (regenerated to 0.0005N-HCl)	0.80	5	4.0	10	6.8
Wofatit M (fully regenerated)	2.15	5	6.2	10	9.5
Amberlite IR4 (regenerated to pH 7)	2.10	5	5.0	10	7.8
Zeo-Karb 215 (acid form)	2.20	5	2.2	10	2.6

The curves in Fig. 3 may be used in the same way as those in Fig. 3 (Part 1) for the calculation of the concentration at which one acid is displaced by another. Inspection of the curves shows that in order to displace aspartic acid as an 0.05M solution by means of a mineral acid, a concentration of about 0.25N-HCl or 0.40N-H₂SO₄ would be required. The high concentrations of mineral acids required must be regarded as a disadvantage in chromatographic experiments, since they increase the risk of chemical degradation of the resin with consequent release of nitrogen-containing compounds, and also tend to increase the swelling suffered by the resin. For this reason lactic acid was used as the displacement developer in some of the displacement experiments, but this was of doubtful advantage since in one experiment bacteria were observed to be growing on the top part of the column in the presence of lactic acid.

The separation of aspartic acid, glutamic acid and serine. It has already been pointed out that the separation of serine from glutamic acid is of importance in the fractionation of the amino-acids of protein hydrolysates, since these two amino-acids form a mixed band when displaced from Zeo-Karb 215 by means of dilute ammonia solution. The separation of serine from glutamic acid should proceed readily on De-Acidite B since serine is adsorbed to a very small extent on this resin. Aspartic acid and glutamic acid may be separated fairly readily on cation-exchange resins when serine is present, but it was nevertheless considered useful to investigate the behaviour of the two acidic amino-acids on an anion-exchange resin.

Fig. 4a gives the results of a separation experiment in which serine (2.3 mmol.), glutamic acid (2.3 mmol.) and aspartic acid (2.3 mmol.) were dissolved in water (75 ml.) and applied to a column of De-Acidite.

The column was 11.5 mm. in diameter and contained 10.66 g. air-dried De-Acidite (dry wt. 6.97 g.). The resin had been treated alternately with N-NaOH and 0.1N-HCl and regenerated with N-NaOH followed by washing with distilled water until the specific conductivity of the effluent fell below 10 gemmhos. After washing, the resin occupied a height in the filtration tube of 15.6 cm. The displacement developer used was 0.101M-lactic acid (concentration as determined by titration with alkali), and this solution was applied at a rate of about 75 ml./hr. Since approximately 130 ml. lactic acid solution were required to saturate the column, this rate of flow corresponded with a rate of progression of the lactic acid boundary down the column of 9 cm./hr. The effluent was collected in 10 ml. fractions, and a qualitative analysis of each fraction was obtained by setting up one-dimensional filter-paper chromatograms (Consden, Gordon & Martin, 1944).

The results are given in the form of block diagrams in Fig. 4a. The figure shows that serine was well separated from the two acidic amino-acids and was retarded by the column to a small extent only. A partial separation of aspartic from glutamic acid was obtained, but the separation was not sufficiently complete to have much practical value. From an analysis of the block diagram, the width of the boundary (λ_{90}) on the column between aspartic acid and glutamic acid was about 6.8 cm., and, since the rate of progression of the front was 9 cm./hr., the width of the aspartic-glutamic boundary was not greater than would be expected for a single-solute front travelling down the column at the same rate (Fig. 2, curve A).

Fig. 4. (a) Separation of serine, glutamic acid and aspartic acid by displacement with 0.10 M-lactic acid ($S = 9$ cm./hr.). (b) Separation of aspartic acid and glutamic acid by displacement with 0.10 M-lactic acid ($S = 1.8$ cm./hr.). (c) Separation of serine and glutamic acid by displacement with 0.51 N-HCl.

This indicated that an improvement in the separation should be obtainable at low rates of progression of the boundaries. To test this a second experiment was set up using the same column. A mixture of aspartic acid (0.306 g.) and glutamic acid (0.339 g.) in water (80 ml.) was applied to the column and a solution of the same concentration (0.101 M) of lactic acid was used as displacement developer. The application of the developer was adjusted to give a rate of progression of the lactic acid boundary of 1.8 cm./hr. The result of the experiment is shown in Fig. 4b, inspection of which shows that the overlap between the aspartic and glutamic acid bands was considerably reduced. However, calculation of the width of the aspartic-glutamic acid boundary shows this to be about 3.0 cm., and thus, at the low rate of flow, the boundary width was greater than the width of a single-solute boundary (1.60 cm.) given by extrapolation of curve A (Fig. 2) to $S = 1.8$ cm./hr.

The results of these two experiments, taken together with those from confirmatory experiments

on the same lines, indicate that the separation of glutamic acid and aspartic acid can only be successful when carried out very slowly, and for this reason it was considered that for practical purposes the method would be of doubtful value, particularly as the two acidic amino-acids are readily separated on cation-exchange columns. The use of the anion-exchange resins, however, provides the simplest method for separating serine from glutamic acid.

Procedure for the regeneration of De-Acidite. The difficulty of washing the commercial anion-exchange resins free from electrolytes after they have been regenerated with strong alkalis has been emphasized already. For experiments with small columns the consumption of distilled water used in the washing procedure after regeneration with sodium hydroxide solution is unimportant, but for larger columns the quantity of distilled water required becomes prohibitive. Nevertheless, complete removal of alkali is necessary for all separations involving weak acids, since its presence raises the pH of the solution in contact with the resin, and thus tends to inhibit the adsorption of the acid. Effects of this sort were met with in experiments with glutamic acid, which failed to form sharp fronts on columns that had been insufficiently washed after regeneration with sodium hydroxide.

Various alkalis and buffer solutions were investigated for use in the regeneration of the resins. The use of sodium carbonate or bicarbonate was undesirable since the evolution of carbon dioxide during the regeneration process necessitated the repacking of the column before each experiment. Attempts were made to use various buffer solutions of pH 8–10, but these also were rather unsatisfactory since the removal (by washing with water) of the excess of electrolyte over this pH range proved to be a slow process. It therefore appeared to be desirable to avoid the use of buffer solutions more alkaline than pH 7, and regeneration with sodium acetate solution was finally adopted as a routine procedure. Details of the procedure are illustrated in the following example.

A column of De-Acidite (125 g. dry wt.; 80–100 mesh/in.) contained in a filtration tube 3.62 cm. in diameter occupied a height of 26 cm., and had been saturated with 0.25N-HCl. A solution of sodium acetate (0.25N; 6 l.) was then passed slowly through the column until the effluent solution reached pH 6 (time 4 hr.). The column was then washed with distilled water (10 l.) until the conductivity of the washings fell to 25 gemmhos. At this point the wash water had an acid reaction (pH 4.5), but was free from chloride.

Separation of serine from glutamic acid on a larger scale. The column used contained 125 g. (dry wt.) De-Acidite B (80–100 mesh/in.) and was 3.62 cm. in diameter and 26 cm. in height. The resin had been

regenerated with sodium acetate solution as described in the previous section. A solution of L-glutamic acid (8.0 g.) and DL-serine (1.50 g.) in water (1 l.) was allowed to flow through the column, and the effluent solution was collected in 93 ml. samples by means of an automatic fraction collector (Brimley & Snow, 1949). The time required for this operation was 4.3 hr. After washing with a small quantity of water (25 ml.) the reservoir was filled with 0.51N-HCl (2 l.), and the solution allowed to flow through the column at a rate of 140 ml./hr. The conductivity of the effluent was measured at intervals throughout the run, and the experiment was stopped when the rise in conductivity showed that the hydrochloric acid boundary had broken through (time 10 hr.; vol. of HCl solution 1.4 l.).

The pH of each 93 ml. fraction was measured and a small quantity of solution was taken from each for chromatographic analysis by the filter-paper method. The chromatogram obtained is illustrated in Fig. 4c, which shows that serine was well separated from glutamic acid. The serine solution emerged from the column at pH 5.4, while the glutamic acid band emerged at pH 3.35, except for the last three fractions which were mixed with hydrochloric acid.

Since hydrochloric acid at a concentration of 0.51N displaces L-glutamic acid at a concentration higher than its solubility in water (Fig. 3) glutamic acid crystals appeared in the effluent solution soon after the fractions were taken. The serine solution (fractions 2–14 inclusive) contained a small concentration of acetic acid derived from the sodium acetate used to regenerate the column. The solution was concentrated under reduced pressure, treated with a small amount of charcoal and finally evaporated to dryness under vacuum at room temperature (yield 1.5 g. colourless crystals: N, found, 13.2%; theory, 13.3%). The glutamic acid solution was collected in two parts; fractions 20–24 inclusive (free from hydrochloric acid) and fractions 25–27 (containing a fairly high concentration of hydrochloric acid). Both portions were evaporated to dryness after treatment with a little charcoal. Fractions 20–24 yielded glutamic acid as a colourless crystalline product (4.73 g.: N, found, 9.9%; theory, 9.5%). The residue from fractions 25–27 was weighed as glutamic acid hydrochloride (pale yellow crystals, 2.2 g.). Total yield calculated as glutamic acid 6.5 g.; recovery 81%.

The separation of glutamic acid from aspartic acid by development with acetic acid. Drake (1947) described an interesting analytical method for the separation of glutamic acid from aspartic acid which could be carried out with a few mg. of material. A column of Amberlite IR4B was used, and the separation was carried out by development with acetic acid, which is adsorbed on anion-exchange resins approximately to the same extent as glutamic,

but less strongly than aspartic acid. With the concentration of acetic acid used (0.05 %) the development results in the formation of an acetic acid band lying between and overlapping the two amino-acid bands, and, since the complete elution of aspartic acid requires a large volume of acetic acid, Drake introduced a second developer (N-HCl) in order to displace the aspartic acid at a suitable concentration.

In view of the rather poor results obtained in the separation of glutamic acid from aspartic acid by the direct application of displacement development, it was considered worth while to investigate an adaptation of the method of Drake to preparative separations involving relatively large quantities of amino-acids. Details of a typical separation experiment are given below.

The column contained De-Acidite B (80–100 mesh; dry wt. 125 g.) and was 26 cm. high $\times$ 3.62 cm. in diameter. The resin had previously been regenerated with sodium acetate solution and washed thoroughly with distilled water. A solution (400 ml.) containing glutamic acid (4.0 g.) and aspartic acid (1.0 g.) was passed through the column (time, 3 hr.) and development was carried out with 0.102N-acetic acid (1.3 l.; time, 8 hr.). The reservoir containing the acetic acid was then replaced by one containing 0.25N-HCl, and this solution (2.5 l.) was allowed to flow into the column for a further 8 hr.

Fig. 5. Separation of glutamic acid from aspartic acid on De-Acidite B. The column was developed with 0.1N-acetic acid followed by 0.25N-HCl.

The effluent solution was collected in 172 ml. fractions, and a one-dimensional paper chromatogram was made from samples taken from each fraction. The chromatogram is illustrated in Fig. 5 which shows complete separation between the aspartic and glutamic acids. The positions of the acetic and hydrochloric acid bands (found by titration) are also indicated in the diagram. It will be observed that the acetic acid front coincided with that due to glutamic acid, while the aspartic acid band was to some extent overlapped by the hydrochloric acid band. Fractions 9–16 inclusive (Fig. 5) were mixed and the solution concentrated under reduced pressure and evaporated to dryness *in vacuo* over sodium hydroxide. The product (glutamic acid) was a colourless crystalline solid (yield 3.27 g.; N,

found, 9.8 %; theory, 9.5 %). The yield of glutamic acid represented a recovery of 82 %. Thus, although separation was complete, there was a loss of 0.73 g. of glutamic acid. In the experience of the authors, losses of this order are usual with both aspartic and glutamic acid where commercial anion-exchangers are employed, and it is considered that the poor yields may in part be due to secondary adsorption of a difficultly reversible character. Chemical degradation of the amino-acids in contact with the resins may also be a contributory factor.

DISCUSSION

In the work described above, three commercial anion-exchange resins were investigated with particular reference to their suitability for the purpose of displacement chromatography on a preparative scale. The two acidic amino-acids, aspartic acid and glutamic acid, were well adsorbed by the resins, and the main object of the work was to establish suitable conditions for the separation of these two substances from each other and from mixtures containing neutral amino-acids. The procedures adopted were based upon those developed for the separation of bases and amino-acids on cation-exchange resins (Partridge & Westall, 1949).

The titration curves of the commercial anion-exchange resins show that they are markedly poly-functional in behaviour; they contain a range of basic radicals of varying strength and also show a weakly acidic character. When in equilibrium at reactions near neutrality they behave as ampholytes, and, for this reason, regeneration of the resins must be carefully controlled. If strong alkalis are used in the regeneration process, cations are adsorbed by the resins which are difficult to remove completely by subsequent washing. Failure to effect the complete removal of alkali after the regeneration process gives rise to a reduction in the adsorptive capacity of the resin for weak acids, since the residual cations become involved in salt formation with the acids, and this increases the pH of the solution in contact with the resin. For this reason, regeneration with strong alkalis should be avoided when dealing with large columns. De-Acidite B may be regenerated effectively with sodium acetate solution, and with this reagent only small volumes of wash water are required to render the columns free from cations and from anions other than acetate.

The anion-exchange resins at present available commercially are relatively weak bases (containing aliphatic and aromatic amino groups as the most basic radicals), and, probably for this reason, the exchange reaction between anions is much slower than that taking place between cations on the strongly acidic resin Zeo-Karb 215. This slowness of reaction is reflected in the broad boundaries shown

by the bands on columns packed with the anion-exchange resins, and measurements of boundary widths at different rates of flow showed that the separation of weak acids by displacement chromatography could only be successful at very slow rates of flow, and by the use of the resins in a finely ground condition.

A number of trial separations using mixtures of aspartic and glutamic acids indicated that, while separations of about the expected order of efficiency were in fact obtained, the rate of flow required was very much slower than for the separation of the same pair of amino-acids on Zeo-Karb 215. Taking the evidence as a whole, it is considered doubtful if the direct application of the displacement technique to the separation of weakly acidic substances on the anion-exchange resins available at present is of practical value.

A particularly useful feature of the resins is that the adsorption of neutral amino-acids is very small, and columns packed with the resins may be used as simple adsorptive filters in order to remove aspartic or glutamic acid from mixtures containing the neutral amino-acids. The separation of glutamic acid from serine and threonine is of particular importance in the fractionation of protein hydrolysates since these three amino-acids form a mixed band on Zeo-Karb 215. The separation of serine from glutamic acid was carried out using De-Acidite B and the experiment gave a quantitative recovery of serine, but a loss of about 20% of the glutamic acid was observed. Losses of this order are a general feature of the adsorption of the acidic amino-acids on the anion-exchange resins, and it is considered that the effect is mainly due to the irreversible adsorption of part of the material. However, a further explanation of the loss may lie in the occurrence of oxidative degradation on the surface of the resin particles.

Although the direct application of the displacement technique to the separation of aspartic and glutamic acid was unsatisfactory, it still remained possible that a combination of elution and displacement would give satisfactory results as a preparative method. Such a method was developed as a microanalytical technique by Drake (1947) who, by developing a column of Amberlite IR4 with dilute acetic acid, was able to effect a complete separation between the two amino-acids. Acetic acid is adsorbed to roughly the same extent as glutamic acid, but is less strongly adsorbed than aspartic acid, and development with this reagent results in the separation of the two amino-acid bands by the interpolation of an acetic acid band between them. An adaptation of this method, using De-Acidite B, gave good results, and a complete separation was obtained using a mixture containing 5 g. of the amino-acids on a column containing 125 g. of the resin.

The results of the investigation as a whole show that the anion-exchange resins at present available are much less efficient for the purpose of displacement chromatography than are resins of the sulphonated phenol-formaldehyde type. This may in large part be due to the polyfunctional character of the anion exchangers and to the rather weakly basic character of the main functional groups. However, the resins may be used with success for the separation of aspartic and glutamic acid from mixtures containing the neutral amino-acids.

SUMMARY

1. Three commercial anion-exchange resins have been investigated for their suitability for displacement chromatography on a preparative scale. These were Amberlite IR4, Wofatit M and De-Acidite B.

2. Titration curves for the three resins are given. These were carried out in the presence of neutral salts and show that the resins have weakly acidic properties in addition to their main function as insoluble bases.

3. The boundary widths given by glutamic acid on columns packed with the anion exchangers are approximately inversely proportional to the adsorptive capacity of the resins. The boundaries given by the anion-exchange resins are much broader at useful rates of flow than those given by the cation exchanger Zeo-Karb 215. This indicates that the exchange reaction is much slower in the former than in the latter case.

4. Some separation was obtained between glutamic acid and aspartic acid on De-Acidite B, but very slow rates of flow were required to give useful results, and (with the anion-exchange resins at present commercially available) the technique is of doubtful value for practical purposes.

5. The anion-exchange resins are, however, useful for the separation of glutamic or aspartic acid from neutral amino-acids, particularly serine. In this case, since the resins adsorb neutral amino-acids to a small degree only, separation may be achieved by application of a simple process of adsorptive filtration.

6. Using De-Acidite B, glutamic acid and aspartic acid may be separated on a preparative scale by developing the column with acetic acid; this forms a band lying between and overlapping the bands due to the amino-acids.

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Displacement Chromatography on Synthetic Ion-exchange Resins

3. FRACTIONATION OF A PROTEIN HYDROLYSATE

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In the first two parts of this series (Partridge & Westall, 1949; Partridge & Brimley, 1949) the application of displacement chromatography to the separation of simple mixtures of bases, acids and ampholytes has been described; columns packed with ion-exchange resins were used and most of the experiments were concerned with two typical commercial products, the cation exchanger Zeo-Karb 215 and the acid-adsorbing resin De-Acidite B. Interest has been particularly directed towards the problem of separating mixtures of amino-acids, since these frequently occur in biological extracts of all kinds, and are almost certain to be met with in any attempt to isolate an active biological substance from its source.

An obvious application of the technique lies in the isolation of the optically active amino-acids from the hydrolysis products of proteins. This problem has been of importance to biochemists in the past, and, in view of the advent of the isotope-dilution technique, the successful development of a simple physical method for carrying out such isolations is likely to be of even more importance in the future. It was considered, furthermore, that an attempt to fractionate the products of hydrolysis of a typical animal protein would present the most practical test of the method, and at the same time would yield valuable information concerning the degree of separation and the order of displacement of a wide range of naturally occurring amino-acids.

A short account of the fractionation of a protein hydrolysate by displacement development on ion-exchange columns has already been given (Partridge, Westall & Bendall, 1947). In recent years much

work has been published on the isolation of amino-acids from protein hydrolysates by the use of ion-exchange resins in various ways, but since several excellent reviews are available (Martin & Synge, 1945; Cannan, 1946; Tiselius, 1947) no attempt will be made to refer to the original publications in detail. However, most of the methods hitherto employed involve the use of the ion-exchange resins as media for elution experiments of the classical type or in simple adsorptive filters. So far as the authors are aware, no systematic attempt to use the displacement technique for this purpose has appeared, although simple mixtures of amino-acids have been separated in this way (cf. Drake, 1947; Hems, Page & Waller, 1948).

Since commercial egg albumin was readily obtainable, and its amino-acid constitution fairly well known, the product of hydrolysis of this protein with hydrochloric acid was chosen for study. Preliminary experiments showed that the amino-acids tyrosine and phenylalanine behaved irregularly on a column of Zeo-Karb 215, presumably because of the intervention of physical (van der Waals) adsorption by the resin material, which is predominantly aromatic in character. However, since the two aromatic amino-acids are strongly adsorbed by charcoal, and may be eluted from it (Schramm & Primosigh, 1943; Tiselius, 1947) it was possible to remove these two amino-acids from the hydrolysate before proceeding with the separation of the remaining constituents on the cation-exchange column. The procedure was found to be particularly advantageous, since in addition to the recovery in a pure condition of tyrosine and phenylalanine, the process