The quantitative separation of amino acids by chromatography on columns of starch has been described in previous publications (1-3). In extending these investigations, a systematic study has been made of the separations of amino acids by elution analysis on columns of synthetic ion exchange resins. With a sulfonated polystyrene resin, Dowex-50, it has been possible to develop a chromatographic technique applicable to a wider variety of problems of biochemical interest. Starch columns possess the disadvantage that fluids of high salt content, such as blood plasma or urine, require desalting prior to chromatography (4). Moreover, contamination of the effluent by traces of carbohydrate, coupled with the relatively low capacity of these columns, tends to interfere with the isolation of pure compounds (5). Methods in which columns of Dowex-50 are employed have been found to be free from these difficulties.

Ion exchange resins of various types have been used in the past to separate amino acids into groups, acidic, basic, and neutral, and also to separate some of the individual basic or acidic amino acids (cf. (6)). The data of Englis and Fiess (7) indicated that certain of the monoamino acids are adsorbed to different degrees by sulfonic acid type resins, but little information was available as to whether columns of ion exchange resins could yield a degree of resolving power comparable with that obtainable with starch or paper. Recently Partridge and coworkers (8-11) have employed displacement development on ion exchange columns for the separation of amino acids on a preparative scale. Those primary fractions containing more than one amino acid were rechromatographed. Their experiments, together with our results by elution analysis (4'), have demonstrated that synthetic resins are capable of separating most of the common amino acids from one another. For analytical experiments, and for isolation work on a small scale, elution analysis would appear to possess higher resolving power and, for this reason, has been employed in the present studies.

In Fig. 1 is shown the result obtained upon passage of a complex mixture of amino acids through a 0.9 × 100 cm. column of Dowex-50. This particular resin (Bauman and Eichorn (12)) is one which is available com-
commercially in a finely divided form well suited for chromatography and has been demonstrated to yield columns of high efficiency for the separation of inorganic cations (cf. (13)). In the experiment shown in Fig. 1, the resin is employed in the sodium form and the amino acids are eluted by

![Graph](image-url)

**Fig. 1.** Separation of amino acids from a synthetic mixture simulating the composition of a protein hydrolysate. The column of Dowex-50, 0.9 × 100 cm., was operated in the sodium form, with buffers of the pH and temperature indicated as eluants. A sample of about 6 mg. of amino acids (see Table II) was chromatographed. The position of an amino acid peak is reproducible, on the average, to better than 5 per cent.
buffers of progressively increasing pH. The results are similar in many ways to those previously reported (5) for columns of Dowex-50 operated in the hydrogen form, from which amino acids were eluted with hydrochloric acid of increasing normality. Although acids as eluants may be useful in isolation experiments, buffers as eluting agents possess several advantages. Many sensitive compounds may be chromatographed with less danger of decomposition when buffers are employed. In addition, a more neutral effluent is easier to analyze by the ninhydrin method.

As may be seen in Fig. 1, it has been possible to arrive at a combination of conditions which, in a single experiment, causes each of the common amino acids to emerge as a discrete peak. In addition, all the amino acids are recovered quantitatively with the exception of histidine, lysine, and arginine. In order to obtain quantitative recovery of the basic amino acids, it has been found necessary to employ a shorter Dowex-50 column and to elute with buffers below pH 7 (Fig. 2).

It is clear that the separations attained in Fig. 1 cannot be ascribed solely to differences in the ionic natures of the amino acids. The rate of travel on a column of a sulfonated polystyrene resin is a function both of the charge possessed by an amino acid and the nature of its side chain; it is a result of the affinity of the resin for both the ionic and the non-ionic portions of the molecule. Accordingly, columns of Dowex-50 exhibit a resolving power for mixtures of amino acids which compares favorably with that obtained on starch. The procedure employed to obtain curves of the types shown in Figs. 1 and 2 is outlined in the experimental section, followed by a discussion of the influence of variables upon the process and
notes on the applicability of the technique to the analysis of protein hydrolysates and of naturally occurring mixtures of amino acids.

**Procedure**

*Preparation of Ion Exchange Columns*—The chromatograph tubes used in these experiments were of the Zechmeister-Cholnoky type, with ground joints and coarse sintered glass plates, and had an inner diameter of 0.9 cm. The tubes for columns of resin 100 cm. in height (Fig. 1) are jacketed to permit temperature control by circulation of water from a constant temperature bath. The jacket, similar to that of a condenser, is 108 cm. long and is constructed of 2 cm. (outer diameter) tubing. The inlet should be about 2 cm. from the bottom of the jacket and the outlet about 4 cm. from the top. The jacket is fastened to the chromatograph tube with rubber stoppers. The shorter columns (15 cm. in height, Fig. 2) were operated without a jacket in a room maintained at $25^\circ \pm 0.5^\circ$.

Dowex-50 (250 to 500 mesh) was purchased from the Microchemical Specialties Company, Berkeley 3, California. 1 pound of the moist resin, as supplied in the hydrogen form, will suffice for the preparation of four 100 cm. columns, with some to spare. The resin (1 pound) is first washed with 4 N HCl on a Büchner funnel with very gentle suction. After washing with 4 to 8 liters of acid, the filtrate, which is initially yellow, should be nearly colorless. After two washes with distilled water (colloidal material may appear in the filtrate), the resin is washed with 2 N NaOH until the filtrate is alkaline. The resulting sodium salt of the resin is suspended in about 3 times its volume of N NaOH and heated over a steam bath for about 3 hours with occasional shaking. The supernatant fluid is decanted or siphoned after about a ½ hour period of settling and replaced with fresh hot N NaOH. This procedure is performed five times in all. The initial supernatant liquids appear very cloudy; the final washes are almost clear. The resin is filtered, washed free of alkali, and passed through a 120 mesh screen with 6 to 8 liters of distilled water. By this procedure a few large particles may be removed. The resin is filtered and stored as the moist sodium salt.

For the preparation of a column, the resin is washed on a filter with a

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1 Purchased from the Scientific Glass Apparatus Company, Bloomfield, New Jersey, catalogue No. J-1663. A length of 115 cm. above the sintered plate must be specified for use with columns 100 cm. in height. The fragility of these long tubes is decreased if heavy walled glass tubing (0.9 cm. inner diameter, 1.5 to 2 mm. wall thickness) is employed in their construction. Tubes 0.9 × 25 cm. are used for the 15 cm. columns. The delivery tip of the chromatograph tube should be beveled.

2 A constant temperature bath, the temperature of which can be varied readily and which is equipped with a circulating pump (E. H. Sargent and Company, type NS1-33 pump, for example), may be employed.
small amount of 0.1 M sodium citrate buffer, pH 3.4 (not containing the detergent used later), and suspended in about 2 volumes of the buffer. The thickness of the slurry should be such that, when the resin is allowed to settle completely, about 200 cc. of supernatant buffer are present per 100 cc. of settled resin. All bubbles should be removed from the slurry by gentle stirring after it has been allowed to stand for an hour or two. To obtain uniformly efficient 100 cm. columns it has been found desirable to pour the slurry into the chromatograph tube in five portions of approximately equal volume (about 50 cc.). Each portion is poured in through a funnel, the tip of which is bent to direct the stream against the side of the tube. The first portion of resin is allowed to settle under air pressure3 of 10 to 15 cm. of mercury until no further drop in the height of the surface of the resin occurs. The pressure is maintained until the liquid has fallen to within about 10 cm. of the surface of the resin, whereupon a second portion of the slurry is added. The final additions (or withdrawals) are made to bring the height of the column to 100 cm. The 15 cm. columns are similarly poured in one or two sections. If, at this stage or later, the top surface of the resin is irregular, it should be stirred with a glass rod in the presence of a few cm. of supernatant buffer and allowed to settle again. A separatory funnel (about 300 cc. capacity) containing an appropriate buffer solution is mounted (cf. (1)) over the column. The funnel may be attached directly, or, with the aqueous solvents used in the present experiments, a length of plastic tubing may be interposed between the funnel and the column. In the latter case, pressure can be adjusted by moving the funnel as a leveling bulb. After about 100 cc. of buffer have been run through, the column is ready for use.

Preparation of Buffers—The composition of the buffers is given in Table I. Since the reproducibility of results on ion exchange columns is dependent upon pH, the pH values of the buffers have been determined accurately. The measurements have been made at 25° ± 0.5° on a model R pH meter manufactured by the Cambridge Instrument Company, Inc., Ossining, New York. A 0.05 M solution of potassium acid phthalate, pH

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3 Accurate control of air pressure has been obtained by the use of equipment manufactured by The Moore Products Company, H and Lycoming Streets, Philadelphia 24, Pennsylvania. The laboratory compressed air (initially at 65 pounds pressure) is passed through a filter (No. 2306) and a reducing valve (No. 60) set to give 20 pounds pressure on a gage (No. 1160-1). Air of the desired pressure for each chromatogram is withdrawn from this 20 pounds line through a Nullmatic pressure regulator (model 40-15), one being employed for each column. The Nullmatic pressure regulators are equipped with the same 0 to 30 cm. of mercury outlet pressure gages described previously (1). In order to prevent clogging of the pressure-regulating equipment by rust, should the air filter become overloaded, it is advantageous to use brass or copper piping on the clean side of the filter.
### TABLE I

#### Preparation of Buffers

<table>
<thead>
<tr>
<th>pH</th>
<th>Composition*</th>
<th>HCl or NaOH required to bring 1 cc. buffer to pH 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.42 ± 0.01 (Fig. 1)</td>
<td>500 cc. pH 5 sodium citrate† + 110 cc. 1.0 N HCl + 390 cc. water + 0.5 cc. thiodiglycol per 100 cc.</td>
<td>0.1 cc. N NaOH</td>
</tr>
<tr>
<td>4.25 ± 0.05 (Fig. 1)</td>
<td>500 cc. pH 5 sodium citrate† + 50 cc. N HCl + 450 cc. water + 0.5 cc. thiodiglycol and 1.0 cc. benzyl alcohol† per 100 cc.</td>
<td>0.05 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>6.7 ± 0.1 (Fig. 1)</td>
<td>21 gm. citric acid monohydrate + 290 cc. N NaOH to volume of 1 liter</td>
<td>0.05 &quot; 0.5 N HCl</td>
</tr>
<tr>
<td>8.3 ± 0.05 (Fig. 1)</td>
<td>250 cc. 0.2 M NaHCO₃,</td>
<td></td>
</tr>
<tr>
<td>9.2 ± 0.05 (Fig. 1)</td>
<td>500 cc. 0.2 M NaHCO₃ + 90 cc. 0.2 N NaOH</td>
<td>0.1 &quot; 2 &quot; &quot;</td>
</tr>
<tr>
<td>11.0 ± 0.05 (Fig. 1)</td>
<td>0.1 M Na₂CO₃</td>
<td>0.1 &quot; 2 &quot; &quot;</td>
</tr>
<tr>
<td>5.0 ± 0.1 (Fig. 2)</td>
<td>500 cc. pH 5 sodium citrate† + 500 cc. water + 0.1 gm. disodium versenate¶ and 1.5 cc. benzyl alcohol per 100 cc.</td>
<td>None</td>
</tr>
<tr>
<td>6.8 ± 0.03 (Fig. 2)</td>
<td>500 cc. 0.1 M Na₃HPO₄ + 450 cc. 0.1 M NaH₂PO₄·H₂O + 0.1 gm. disodium versenate¶ and 1.5 cc. benzyl alcohol per 100 cc.</td>
<td>0.05 cc. 0.5 N HCl</td>
</tr>
<tr>
<td>6.5 ± 0.05 (Fig. 2)</td>
<td>42 gm. citric acid monohydrate + 580 cc. 1.0 N NaOH to volume of 1 liter + 0.1 gm. disodium versenate¶ and 1.5 cc. benzyl alcohol per 100 cc.</td>
<td>0.05 &quot; 2 N HCl</td>
</tr>
</tbody>
</table>

* To all buffers, 1 cc. of BRIJ 35 solution per 100 cc. is added.
† This is the pH 5 (0.2 M) buffer employed for the ninhydrin method (2). A 1:1 dilution of the buffer normally has a pH of 4.95.
‡ The benzyl alcohol serves to improve the resolution of tyrosine and phenylalanine.
§ Freshly prepared before use.
|| This buffer loses CO₂ on standing and hence becomes more alkaline. Add a 1 cm. layer of mineral oil over the buffer in the separatory funnel to stabilize the pH during the chromatographic experiment.
¶ Analytical grade. We are indebted to the Bersworth Chemical Company, Framingham, Massachusetts, for disodium versenate which contains negligible amounts of ninhydrin-positive material when tested in 0.1 per cent concentration.

4.00, was used as a standard. The various buffers can be prepared in quantity, adjusted if necessary with a few cc. of N HCl or NaOH, and stored in the cold with thymol.

The operation of ion exchange columns with a detergent in the eluant
has been found to permit faster flow rates without concomitant broadening of the peaks on the effluent curves. Therefore, for each 100 cc. of buffer taken for a chromatographic experiment, 1 cc. of a detergent solution is added just before use. The detergent solution is prepared by dissolving 50 gm. of BRIJ 35 (Atlas Powder Company, Wilmington, Delaware) in 100 cc. of water. BRIJ 35 is an ether of polyethylene glycol and has the advantage of being a neutral and relatively stable detergent.

For the 100 cm. columns, the buffers all have about the same sodium concentration (0.2 M), in order to minimize the swelling and shrinking of the ion exchange resin which would occur with changes in the ionic strength of the eluant. Before analysis by the ninhydrin method, each effluent fraction must be brought to about pH 5. The amount of acid or alkali required to make this pH adjustment for a 1 cc. effluent fraction is included in Table I for each buffer. The acid or alkali is added from a burette in the manner already described (3). If acid is allowed to remain in a burette for several hours, the tip of the burette must be wiped free of NH₄Cl before use.

It has been observed with ion exchange columns, as with starch chromatograms (1), that losses of methionine occur unless thiodiglycol is added as an antioxidant. Thiodiglycol can be obtained by the redistillation of Kromfax solvent (Carbide and Carbon Chemicals Corporation 30 East 42nd Street, New York) under reduced pressure.

Since the buffer of pH 3.42 ± 0.01 is the only one the performance of which requires checking, it is convenient to prepare it in 10 liter quantities. To check the buffer, a mixture of alanine, cystine, and valine is chromatographed. The pH of the buffer is then adjusted so that the cystine peak is about midway between the neighboring alanine and valine peaks (Fig. 1). The position of the cystine peak is very sensitive to the pH of the buffer. At pH 3.47 cystine emerges almost with alanine, while at pH 3.37 its peak begins to overlap that of valine.

**Operation of 100 Cm. Columns**—About an hour prior to use, the prepared column is mounted on a fraction collector (1), and water, temperature-controlled at 37.5° ± 0.5°, is started circulating through the jacket. The synthetic mixture of amino acids analyzed in the present work was similar to that employed previously (3). A 0.1 cc. aliquot of the acidic synthetic mixture was added to 1 cc. of the buffer of pH 3.42 and 0.5 cc. of the resulting solution was placed on the column. In the case of more dilute protein hydrolysates, 1 cc. of the hydrolysate can be added to 1 or 2 cc. of buffer, and 1 or 2 cc. sample placed on the column. A pipette with a bent tip facilitates the addition of the sample to the column without disturbing the surface of the resin. A similar pipette is used to add three 0.3 cc. washes of buffer, as described for starch (1), except that the
sample and washings are allowed to drain into the column under gravity. The surface of the resin should not be allowed to remain dry for more than about 5 minutes. A longer time may cause bubbles of air to be trapped in the surface of the column. In adding samples to a buffered column of Dowex-50, it is important that the pH of the sample be the same or less than the pH of the column. A pH of 2.5 to 3.0 is optimum. Brief contact with a buffer of higher pH can cause the bands to spread. A convenient load for analytical work is about 5 mg. of total amino acid mixture (0.1 to 0.5 mg. per amino acid). Since the capacity of Dowex-50 columns is high, the total load can be increased several fold, if desired, to increase the accuracy of the determination of components present in small amounts.

When heated columns are employed, it is necessary to use relatively air-free buffers in order to prevent the formation of bubbles in the column as the temperature is raised. At the beginning of the experiment shown in Fig. 1, about 300 cc. of buffer of pH 3.42 were heated to boiling, poured into the separatory funnel, and while still warm, covered with a layer of mineral oil to prevent access of air. The use of boiled buffer is not essential as long as the column is operated at 37.5°. Since, however, it is necessary to have only air-free buffer of pH 3.42 in contact with the column before the temperature is raised to 50°, it is usually convenient to use the boiled solution from the beginning.

The rate of solvent flow through the column should be adjusted to about 4 cc. per hour. A faster rate will cause the peaks to broaden, particularly in the leucine-isoleucine range (Fig. 1). The 100 cm. columns usually run at the appropriate rate under gravity or slight pressure. The effluent is collected in 1 cc. fractions. The photometer tubes employed on the fraction collector do not have to be coated with a silicone film (3) when aqueous eluants are employed. Solvent changes are made in the manner previously described (3). The boiled buffer of pH 4.25 should be run on to the top of the column without letting the surface run dry, that is, while 1 mm. of the previous buffer is still present.

The change of solvent to buffer of pH 4.25 should take place at the beginning of the valine peak (about 225 cc.). During the course of an experiment, the time of change can best be estimated by multiplying the observed position of the proline peak by 1.63. The temperature is also changed at this time to 50°. As the temperature rises, the rate of flow will increase and a lowering of the pressure is required to maintain the desired rate of flow. 75 cc. later (at about 300 cc.) the temperature is increased to 75° to improve the separation of tyrosine and phenylalanine. Frequently the chromatogram is stopped at 375 cc., which is the end of the quantitative part of the experiment. If stopped, the column should be allowed to cool to room temperature before closing the stop-cock of the
separatory funnel. If the chromatogram is to be continued for elution of the basic amino acids, subsequent operation is at 25°, with changes of eluant to buffers of pH 6.7, 8.3, 9.2, and 11, as indicated in Fig. 1. The buffer of pH 6.7 is interposed for a 5 to 6 hour period between the buffers of pH 4.25 and 8.3 to avoid the generation of bubbles of CO₂ in the column which might otherwise occur were the shift to be made directly. After the experiment is finished, the column should finally be cleaned with about 100 cc. of 0.2 N NaOH containing BRIJ 35, which may be run through rapidly if necessary. The column is then ready for conversion to pH 3.42 and reuse.

Dowex-50 columns can be used over again indefinitely, so far as we have observed. If not used promptly, the top and bottom of the chromatograph tube should be closed off to prevent drying of the resin. If the surface of the column becomes clogged in the course of use, the top cm. of the adsorbent should be removed and replaced by fresh resin.

Operation of 15 Cm. Columns—The shorter columns employed for the determination of the basic amino acids (Fig. 2) are operated at 25°. The pH of the sample to be analyzed should be about 4. The 15 cm. columns run too rapidly under gravity and require a slight negative pressure, which may be applied by pulling air through a water tower connected to the top of the separatory funnel by rubber tubing, or by lowering the separatory funnel itself. The shifts of buffer are made from pH 5 to 0.1 M phosphate, pH 6.8, and 0.2 M citrate, pH 6.5, as indicated in Fig. 2. If histidine emerges too close to lysine, a phosphate buffer of pH 6.85 or 6.90 may be employed. The column is cleaned with 0.2 N NaOH before reuse.

Analysis of Effluent Fractions—The effluent fractions are brought to about pH 5 with one or two 0.05 cc. drops of the appropriate concentration of HCl or NaOH and analyzed by the photometric ninhydrin method (2). The hold up volume of the 0.9 X 100 cm. columns is about 22 cc. The more acidic buffers, however, have a significant retardation volume. Hence, the amount of NaOH added to each fraction should be decreased from 0.1 to 0.05 cc. at a point 35 cc. after the buffer of pH 4.25 is added to the top of the column. The subsequent changes in neutralization of the effluent fractions are made at a point 25 cc. after the buffers in question have been added to the column. For the 15 cm. columns (Fig. 2), the corresponding changes in neutralization are made 4 cc. after the change of buffer.

Ammonia emerges from the 100 cm. column (Fig. 1) as a peak very near arginine. Since quantitative recoveries of ammonia are not possible,
because of loss by volatilization from the alkaline buffer, it is preferable to remove ammonia completely before analysis. For this purpose, groups of 50 of the tubes containing the buffer of pH 11 are placed in a large vacuum desiccator prior to neutralization, and the desiccator is evacuated continuously by a water pump for 2 hours at room temperature.

In order to obtain accurate analytical values, the 1 cc. effluent fractions require the addition of 2 cc. of ninhydrin reagent and this procedure has been followed in the present work. Nearly the same accuracy can be achieved, however, with 1 cc. of ninhydrin solution, provided a 30 minute heating time is used instead of the usual 20 minutes. With 1 cc. samples and 2 cc. of ninhydrin reagent, the color yields of most of the amino acids have been checked and found to be the same as those obtained before ((2), Table II) with the following changes: aspartic acid 0.93, serine 0.97, alanine 0.99, valine 1.00, and tyrosine and phenylalanine 0.91. The correction factors were calculated according to the equation given previously (2), with the knowledge that no loss in volume because of evaporation occurs during the heating period. For maximum accuracy, it is desirable that the volume of the fractions delivered by the fraction collectors be 1.0 ± 0.1 cc. The volume calculations and the adjustment of the pH are sufficiently sensitive so that a 0.1 cc. variation in the volume of 1.0 cc. effluent fractions can give rise to a 2 per cent error in the final integration.

As has been pointed out previously (1, 3), the choice of blanks against which the amino acid peaks are read is crucial for maximum accuracy. In order to insure a constant blank reading with buffers of pH 3.42 and 4.25, it is essential to take precautions (3) against the uptake of ammonia while the full tubes are on the fraction collector. From Fig. 1 it can be seen that the base-line for the aspartic acid-proline range can be obtained readily from the several valleys in that portion of the curve. The blank after proline can always serve as the point of reference for the glycine and alanine peaks. Depending upon the exact position of cystine, the curve may or may not return to the base-line both before and after the cystine peak. Hence the base-line for cystine and valine can be chosen after valine. The blank reading may change slightly at the point of emergence of the buffer of pH 4.25, just ahead of methionine. The blank for the three peaks in this range is, therefore, best chosen after leucine.

In the determination of the basic amino acids (Fig. 2) the emergence of the buffer of pH 6.5 is accompanied by a rise in the blank of about 0.15 optical density unit. The blank for the ammonia peak should, therefore, be chosen just after the peak rather than before it. The high blank, which results from the mobilization of traces of ammonia adsorbed from the previous buffers, does not prevent accurate integration of the relatively sharp ammonia peak superimposed thereon. The high blank con-
tinues as a plateau under the ammonia peak and for a number of fractions thereafter, but falls again to the usual low value before arginine.

Quantitative Analysis of Synthetic Mixtures—The average results obtained by the integration of the curves in chromatograms of the type shown in Fig. 1 are summarized in Table II. Recoveries of 100 ± 2 per cent are obtained for the majority of the amino acids. Low yields are obtained for the basic amino acids, and slightly low results are given by glutamic acid, cystine, and methionine. In the case of glutamic acid, the

Table II

Recovery of Amino Acids from Known Mixture

The mixture was chromatographed on Dowex-50 in the manner indicated in Fig. 1. Approximately 6 mg. of the mixture were employed for each chromatogram.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Average recovery</th>
<th>Average deviation from mean</th>
<th>No. of chromatograms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>99.6</td>
<td>±2.7</td>
<td>12</td>
</tr>
<tr>
<td>Threonine</td>
<td>101.9</td>
<td>±3.2</td>
<td>12</td>
</tr>
<tr>
<td>Serine</td>
<td>100.4</td>
<td>±2.6</td>
<td>12</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>96.7</td>
<td>±2.1</td>
<td>11</td>
</tr>
<tr>
<td>Proline</td>
<td>101.0</td>
<td>±2.4</td>
<td>11</td>
</tr>
<tr>
<td>Glycine</td>
<td>99.2</td>
<td>±2.8</td>
<td>12</td>
</tr>
<tr>
<td>Alanine</td>
<td>100.7</td>
<td>±2.2</td>
<td>12</td>
</tr>
<tr>
<td>Cystine</td>
<td>96.1</td>
<td>±1.9</td>
<td>10</td>
</tr>
<tr>
<td>Valine</td>
<td>100.8</td>
<td>±2.9</td>
<td>12</td>
</tr>
<tr>
<td>Methionine</td>
<td>95.8</td>
<td>±2.2</td>
<td>11</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>101.0</td>
<td>±1.4</td>
<td>7</td>
</tr>
<tr>
<td>Leucine</td>
<td>100.6</td>
<td>±2.1</td>
<td>12</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>99.8</td>
<td>±2.6</td>
<td>5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>98.2</td>
<td>±1.4</td>
<td>5</td>
</tr>
<tr>
<td>Histidine</td>
<td>70 Ca.</td>
<td>±11 Ca.</td>
<td>9</td>
</tr>
<tr>
<td>Lysine</td>
<td>82 &quot;</td>
<td>±5 &quot;</td>
<td>9</td>
</tr>
<tr>
<td>Arginine</td>
<td>71 &quot;</td>
<td>±6 &quot;</td>
<td>8</td>
</tr>
</tbody>
</table>

yield of 96.7 per cent is constant and is independent of the amino acid concentration. Analytical results for glutamic acid can therefore be corrected for the 3 per cent loss, which is probably the result of pyrrolidone-carboxylic acid formation in the feebly acidic solvent. As would be expected on this assumption, the yield is a function of time and of the temperature at which the column is operated. At 25°, essentially quantitative recoveries of glutamic acid are obtained, whereas with columns operated at 60° the recovery drops to below 70 per cent. The slightly low recovery of methionine may be occasioned by failure of the thiodiglycol to protect the sulfur completely from oxidation, or it may be a reflection of slight impurities in the sample of methionine employed.

No satisfactory explanation can be offered at present for the low recov-
eries of the basic amino acids when alkaline buffers are used as eluants. Experiment has shown that a notable improvement of the recoveries is not obtained if buffers such as phosphate and borate are substituted for the carbonate buffers. The yields are unaffected by the presence of thiodiglycol as an antioxidant or of versene to remove traces of metal ions. Satisfactory recoveries are not obtained if the column is operated at 4° or 37.5°, instead of the usual 25°. The basicity of the amino acid is not a determining factor, since tryptophan, which emerges before lysine (see Fig. 3), is recovered to no greater extent than is arginine. Losses are also encountered with histamine.

It has not been possible to obtain quantitative results for the basic amino acids unless eluting buffers of pH 7 or less are employed. In Fig. 2, the first large peak eluted at pH 5 contains all the amino acids which emerge before tyrosine and phenylalanine in Fig. 1. The recoveries for tryptophan and subsequent peaks are 100 ± 3 per cent under these conditions, as may be seen in Table III. To attain quantitative results, the chromatogram must be started with a buffer of pH 5. If it is started with a buffer of pH 6.8, the results are low, particularly for histidine. If versene is not included, low recoveries may be obtained after continued use of the columns. The benzyl alcohol serves to sharpen the tryptophan peak and to move it ahead of histidine.

Chromatographic Analysis of More Complex Mixtures—In many biological media, ninhydrin-positive substances other than the ones shown in Fig. 1 are encountered. The positions of some amino acids and related compounds not discussed thus far are shown in Fig. 3. Thirty-two substances were chromatographed together and thirty peaks can be recognized on the effluent curve, with two complete overlaps. The positions of the individual components in this case, and in earlier instances, have been established by preliminary chromatograms with simpler mixtures. Cys-

### Table III

Recovery of Amino Acids from Column of Dowex-50 15 Cm. in Height

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Chromatogram 908</th>
<th>Chromatogram 909</th>
<th>Chromatogram 910</th>
<th>Chromatogram 911</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>100</td>
<td>102</td>
<td>104</td>
<td>108</td>
</tr>
<tr>
<td>Histidine</td>
<td>99</td>
<td>98</td>
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<td>Lysine</td>
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<td>Ammonia</td>
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<tr>
<td>Arginine</td>
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emerge before tyrosine and phenylalanine in Fig. 1. The recoveries for tryptophan and subsequent peaks are 100 ± 3 per cent under these conditions, as may be seen in Table III. To attain quantitative results, the chromatogram must be started with a buffer of pH 5. If it is started with a buffer of pH 6.8, the results are low, particularly for histidine. If versene is not included, low recoveries may be obtained after continued use of the columns. The benzyl alcohol serves to sharpen the tryptophan peak and to move it ahead of histidine.
tein, if present, is gradually oxidized and does not yield a peak. The many variations in the nature of the eluting conditions which can be

![Diagram of amino acid separation](image-url)

**Fig. 3.** Separation of amino acids and related compounds from a synthetic mixture containing thirty-two components. The experimental conditions are the same as those given in Fig. 1.

effected with Dowex-50 columns increase the likelihood of resolving substances which are not well separated by the buffer systems employed for the experiment shown in Fig. 3. For example, if a buffer of slightly lower pH, such as 3.2, is employed in place of the buffer of 3.4, hydroxyproline
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will move farther ahead of aspartic acid. On the other hand, a pH of 3.6 will move cystine to a point well ahead of α-amino-n-butyric acid. The importance of applying as many criteria as possible to the identification of the peaks on a complex curve has been stressed for the amino acids in the case of starch (1, 3) and applies with equal emphasis here.

The 15 cm. columns used for the quantitative determination of the basic amino acids do not possess as high a resolving power as do the 100 cm. columns. For example, ornithine emerges with lysine in the experiment shown in Fig. 2 and can only be recognized by the slight asymmetry it confers upon the leading edge of the lysine peak. Hydroxylysine appears well ahead of lysine, but coincides with histidine, while glucosamine yields a sharp peak superimposed upon the center of the broader tryptophan peak. β-Alanine travels very close to tyrosine and phenylalanine. Since the 15 cm. column has limited resolving power, it is recommended that an unknown mixture first be chromatographed in the manner shown in Fig. 1, in order to obtain a qualitative picture of the components in the basic amino acid range.

DISCUSSION

Effect of Variations in Resin—The procedure leading to effluent curves of the type shown in Figs. 1, 2, and 3 has grown out of a study of the characteristics of columns of different preparations of sulfonated polystyrene, operated under varying conditions of pH and with buffers of different anionic and cationic nature, at various flow rates and loads. The nature and constancy of behavior of the resin are, of course, central factors in the problem. Different manufactured lots of the standard Dowex-50 (250 to 500 mesh spheres) have given the same results. A curve similar to the one shown in Fig. 1 was obtained with a "low color" resin prepared by a different process of sulfonation. Major variations in the particle size and the degree of cross-linking of the polystyrene polymers, however, can cause different results. With 100 mesh resin the peaks were about twice as broad as those shown in Fig. 1. The improved performance of finer mesh material has been demonstrated for the separations of the rare earth elements (13, 14). The results of the amino acid studies parallel the observations made with inorganic ions with the exception that, in amino acid chromatography, colloidal agglomerates of Dowex-50 were found to have no advantage over the 250 to 500 mesh beads.

The standard Dowex-50 resin used in this work was prepared from styrene copolymerized with about 8 per cent divinylbenzene. The resulting

5 The different types of Dowex-50 resins discussed in this section were obtained from Dr. W. C. Bauman of The Dow Chemical Company, Midland, Michigan, whose cooperation we wish to acknowledge with gratitude.
product may be considered to have a molecular structure the holes or pores of which are characteristic of 8 per cent cross-linking. When amino acids were chromatographed on a 16 per cent cross-linked resin (250 to 500 mesh) at a rate of solvent flow similar to that used with the 8 per cent resin, broad, very poorly resolved zones were obtained. This result indicates that the pore size of this resin was too small to permit rapid penetration into the resin particle of substances the size of amino acids. If this supposition is correct, there should also be a molecular weight range above which efficient chromatography on the 8 per cent cross-linked resin will not be feasible. In the present work it has been found that dipeptides such as glycylleucine can be chromatographed well on the standard resin. The tetrapeptide, leucylleucylglycylglycine, gave a somewhat broader peak, however. To obtain sharp peaks with the higher peptides, resins of 4 or 2 per cent cross-linking may be required. Lower cross-linked resins are not so satisfactory for amino acid chromatography, however. It has been found, for example, that, compared to the 8 per cent resin, the 2 per cent resin is somewhat deficient in resolving power. Under conditions similar to those shown in Fig. 1, serine and threonine are not well separated, nor are glycine and alanine. In addition, the greater tendency of the 2 per cent resin to shrink and swell with changes in ionic strength gives rise to columns of poorer mechanical stability. The amino acid peaks obtained from columns of the 2 per cent resin are not sharper than those shown in Fig. 1. Moreover, faster rates of solvent flow cause the peaks to broaden just as much on the 2 per cent resin as was found to be the case for the 8 per cent resin. In general, it appears preferable to use a resin with the highest degree of cross-linking compatible with the separations to be achieved.

Effect of Variations in Eluant—The chromatographic behavior of amino acids on columns of Dowex-50 operated in the sodium cycle can be varied by changes in the pH and the ionic strength of the eluting buffers. In general, low pH values decrease and high pH values increase the rate of travel of the amino acids shown in Fig. 1. Thus, at pH 2.5, the amino acids are retained near the top of the column. At pH 5, on the other hand, all except the basic amino acids move rapidly (see Fig. 2). As might be expected, the positions of aspartic acid, glutamic acid, cystine, and proline are relatively the most sensitive to pH when buffers in the range pH 3 to 5 are employed. If, for example, operation of a column is begun with a buffer of pH 4.0, instead of the buffer of pH 3.4 shown in Fig. 1, aspartic and glutamic acids are accelerated preferentially and emerge still separated but ahead of both threonine and serine. Cystine also moves faster and appears before glycine, while proline is relatively retarded and emerges near alanine. Under these conditions, moreover, the resolution of gly-
cine, alanine, and valine is largely lost, and these three emerge very close together. As has already been noted for the case of hydroxyproline and the basic amino acids, the use of buffers possessing a pH different from any employed in the experiments shown in Figs. 1 and 2 can aid in the resolution of specific overlaps.

The effect of variations in the molarity of the citrate buffers has also been studied. In general, raising the Na\(^+\) concentration produces changes similar to those induced by raising the pH. Thus, 0.2 M sodium citrate at pH 3.1 gives a pattern very similar to that obtained at pH 3.4 with the 0.1 M buffer, except that the aspartic acid-threonine and glutamic acid-proline separations are marginal. With buffers of much lower concentration than 0.1 M, unsatisfactorily broad and asymmetric peaks are obtained. The effect of employing buffers of higher ionic strength has been utilized to advantage in the experiment shown in Fig. 2. A more concentrated buffer (0.2 M citrate), rather than one of higher pH, was employed to accelerate arginine.

The properties of the eluant can also be changed by the addition of an organic solvent. For example, the presence of about 1 per cent benzyl alcohol in the eluant has been found to have a sharpening effect on the peaks of the aromatic amino acids and tends to increase their rates of travel relative to the others. Experiments with citrate buffers containing varying amounts of propyl alcohol have revealed that, in general, organic solvents accelerate preferentially the amino acids possessing the longer non-polar side chains, thus, in effect, tending to reverse the normal order of emergence for the glycine through leucine group of amino acids. With 25 per cent propyl alcohol, alanine is moved ahead of glycine, isoleucine overlaps valine, and threonine is farther in front of serine. For specific separations, particularly in preparative work, alcoholic solvents can frequently be used to advantage.

**Effect of Temperature**—The separations of amino acids obtained on columns of Dowex-50 are dependent on temperature. Increasing the temperature of the citrate-buffered columns increases the rate of movement of all the amino acids. Part of this effect results simply from the rise in pH which occurs when citrate buffers are warmed. More specific effects also occur, however. For example, methionine and isoleucine appear together when eluted at 25° under conditions otherwise quite similar to those shown in Fig. 1. At 60°, on the other hand, methionine is well ahead of isoleucine. Tyrosine and phenylalanine are also completely resolved at 60°, whereas at 25° and 37° they emerge together. Operation of the column at 60° throughout has the disadvantages that isoleucine

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6 The effect of temperature upon the separability of methionine and leucine has been reported recently by Partridge and Brimley (11).
and leucine are poorly separated, and that the yield of glutamic acid is markedly lowered, as has been mentioned previously. A consideration of all the factors involved suggested the sequence of temperatures shown in Fig. 1. The column is warmed just enough at the beginning to insure the separation of methionine and isoleucine without impairing the separation of the latter from leucine. After the emergence of isoleucine, the temperature is again raised to facilitate the separation of the two aromatic amino acids. It should be mentioned that, as was found with inorganic ions (13, 14), the rate of the exchange of amino acid ions between solution and resin is probably increased at higher temperatures. For this reason somewhat faster rates of solvent flow can be used under these conditions without a concomitant broadening of the amino acid peaks.

Operation of Dowex-50 Columns in Hydrogen and Ammonium Cycles—An effluent curve showing the results which may be obtained with a Dowex-50 column operated in the hydrogen cycle has already been published ((5), Fig. 8). Prior to analysis by the ninhydrin method, the HCl was removed from the effluent fractions by evaporating them to dryness in a vacuum desiccator attached to a water pump and warmed to 40° with an infra-red lamp or a heating mantle. Alternatively, the samples can be neutralized accurately with NaOH and analyzed directly. The increased volume of the neutralized samples requires the use of more ninhydrin solution, however, unless aliquots are pipetted for analysis. For the isolation of many of the amino acids, the HCl system can be useful (cf. (15)), although operation in the ammonium cycle with ammonium formate, acetate, and carbonate buffers will probably prove preferable. These can all be removed conveniently by sublimation. A systematic scheme permitting the isolation of amino acids from 2 to 5 gm. of a protein hydrolysate by the use of such buffered columns has been developed by Dr. C. H. W. Hirs and will form the subject of a forthcoming communication.

Operation of Dowex-50 in Lithium and Potassium Cycles—When operated at 25° with lithium citrate buffers, Dowex-50 gives a curve very similar to the one shown in Fig. 1. The resolution of some amino acids is slightly improved, but the separation of glycine and alanine is less complete. It is primarily for this reason that operation in the sodium rather than the lithium cycle has been chosen. With the potassium salt of the resin, very poor resolution and markedly broad peaks were obtained.

Applicability of Method—Compared with the starch method (1, 3, 16), the ion exchange technique is about twice as rapid for the analysis of protein hydrolysates. The 100 cm. column (Fig. 1) requires 4 or 5 days to run through tyrosine and phenylalanine. If the 3 day curve for the basic amino acids (Fig. 2) is obtained concurrently, analysis for all the com-
ponents of an acid hydrolysate (cysteine excepted) can be completed in 5 days. The determination of tryptophan in alkaline hydrolysates has not been studied by the present method.

The procedure has been applied to blood plasma and urine. The fact that the performance of the column is not altered by the presence of the inorganic salts of the blood was first established with synthetic mixtures containing the appropriate ratio of salt to amino acid. In practice, the dialysate (17) from 5 cc. of blood plasma can be concentrated and chromatographed exactly in the manner described for protein hydrolysates. In the case of urine, only a slight modification in technique is required. The very large amounts of urea and other constituents in a 4 cc. sample of urine of specific gravity 1.020 cause a distortion of the peaks in the aspartic acid-proline range. This distortion can be eliminated if the column is started with a citrate buffer of pH 2.5. After 60 fractions, a buffer of pH 2.92 is introduced for forty additional fractions, followed by the buffer of pH 3.42 and the usual shifts thereafter. The points of emergence of taurine and urea given in Fig. 3 are not noticeably different in the buffer of pH 2.5. The remaining amino acids move so slowly at the lower pH values that the pattern obtained after the shift to the buffer of pH 3.42 is almost identical with that shown in Figs. 1 and 3. There are a number of peaks on urine curves the full interpretation of which requires further investigation.

The authors wish to acknowledge with appreciation the expert technical assistance of Mrs. Gertrude C. Carey.

SUMMARY

A procedure is described for the chromatographic fractionation of mixtures of amino acids by elution analysis on columns of Dowex-50, a sulfonated polystyrene resin. On a 0.9 X 100 cm. column operated in the sodium form, elution of amino acids is effected by the use of buffers of progressively increasing pH, from 3.4 to 11. In experiments with synthetic mixtures of amino acids simulating the composition of protein hydrolysates, a sequence of buffers has been developed which yields in a single chromatogram an effluent curve in which every component emerges as a discrete peak. For analytical work, 3 to 6 mg. of the amino acid mixture are required per chromatogram. Integration of the curves has given quantitative recoveries (100 ± 3 per cent on the average) for all except the basic amino acids. These latter may be determined with the aid of a second column, 0.9 X 15 cm., with buffers in the range pH 5 to 6.8. The position of emergence of some of the less common amino acids and related compounds has also been determined.
The resin columns possess higher resolving power than starch columns and are more convenient to operate. An amino acid analysis of a protein hydrolysate can be carried out in about half the time required with starch. The performance of the ion exchange column is not adversely affected by the presence of inorganic salts in the material chromatographed. Accordingly, blood plasma dialysates and urine may be fractionated directly without preliminary salt removal.

BIBLIOGRAPHY