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The Chromatography of Amino Acids on Ion Exchange Resins. Use of Volatile Acids for Elution

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For the isolation of pure amino acids from protein hydrolysates, it is advantageous to have available chromatographic systems in which the eluants can be removed by simple evaporation. The method previously outlined in preliminary form, wherein amino acids are eluted from a sulfonated polystyrene resin (Dowex 50-X8) with HCl, has proved useful for this purpose, but the resolving power has not been sufficient to permit isolation of all of the common constituents of protein hydrolysates. In the procedure described in the present communication, aspartic acid, glutamic acid and tyrosine are isolated by passage of the hydrolysate over a 30-cm. column of the acetate form of Dowex 1-X8 (a strongly basic resin) using 0.5 *N* acetic acid as eluant, and the remaining components of the mixture are separated on a 150-cm. column of Dowex 50-X4 by elution with 1 to 4 *N* HCl.

Ion-exchange columns have been utilized in a number of different ways for the isolation of amino acids. The chromatographic methods of high resolving power developed for quantitative analysis[1,2] can be used for preparative purposes if the amino acids are separated from the non-volatile buffer salts by cycling the effluent over cationic or anionic resins.[3,4] For example, the amino acid 3-methylhistidine was isolated from urine in this manner by Tallan, *et al.*[5] Using volatile ammonium formate and acetate buffers, which are removable by sublimation at 40°, a system for the isolation of amino acids has been recently devised,[6] which is also sufficiently mild to be applicable in principle to the preparation of peptides or other substances that might be labile in acid solution. The displacement development methods of Partridge and associates are frequently advantageous, in view of their greater capacity, if the higher resolving power of elution analysis is not required.[6]

When elution analysis is employed, the use of volatile acids as eluants has the advantage that the amino acid can be obtained by simple concentration of the appropriate fractions. Chromatography on columns of Dowex 50-X8 operated with 1 to 4 *N* HCl, in the manner previously outlined in preliminary form,[7] has been adapted to the isolation of isotopically labeled amino acids in several series of investigations (*cf.* Ehrensvärd, *et al.*,[8] Åqvist,[9] Lien, Peterson and Greenberg,[10] Wall[11] and Simpson and Velick[12]). The method, however, does not have sufficient resolving power to permit isolation of all of the common amino acids in pure form, and the present study was undertaken to arrive at a procedure which would have wider applicability. The use of Dowex 50-X8 (200–400 mesh) that has been screened by the user through a 200-mesh sieve[2] will yield results which are superior to those previously published.[7] Recent experience with the sodium salts of resins of different degrees of cross-linking[2] has shown, however, that, with Dowex 50-X8, separations of amino acids are borderline because attainment of equilibrium is not always sufficiently rapid. Sharp peaks at faster flow rates are attainable with Dowex 50-X4. Even under optimum conditions, it has been found in the present studies that the resolution of the four most rapidly moving amino acids (aspartic acid, glutamic acid, serine and threonine) on the acid form of Dowex 50-X8 or -X4 is not sufficient for preparative work. Therefore, a preliminary separation of aspartic and glutamic acids by elution with dilute acetic acid from a basic resin has been introduced as a first step. Tyrosine is also isolated at this stage. The remaining fourteen amino acids, which pass through the basic column without significant retardation, are adequately separated on a 150-cm. column of Dowex 50-X4 operated at 25 and 50°. The separations obtained by this procedure are illustrated in Fig. 1.

When strong HCl is employed as eluant, Dowex 50 columns may contribute more soluble material to the effluent than is encountered with buffered columns.[6] For example, 100 ml. of 4 *N* HCl obtained as effluent from a Dowex 50-X4 column may contain 5 to 10 mg. of a brown residue. This quantity is generally less than the weight of amino acid in the same volume of effluent and may not interfere with radioactivity measurements.[12] The residue from the resin is completely removed in the present procedure, however, when the free amino acid is prepared from the hydrochloride by passage of the concentrated amino acid solution through a small bed of the acetate form of a basic ion-exchange resin. By substituting the free base form of the resin for the acetate, the same ion-exchange step also separates alanine from the ammonia with which it emerges under the conditions used for the experiment shown in Fig. 1.

The effluent from the 30-cm. Dowex 1-X8 column (Fig. 1) is not entirely residue-free, 100 ml. of the 0.5 *N* acetic acid containing about 1 mg. of solids. This small residue does not interfere with the direct crystallization of tyrosine, glutamic acid, and aspartic acid from the effluent.

Experimental

The following section includes only the details required in addition to those given for the operation of buffered Dowex

(1) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).
(2) S. Moore and W. H. Stein, *ibid.*, in press.
(3) A. Drèze, S. Moore and E. J. Bigwood, *Anal. Chim. Acta*, in press.
(4) W. H. Stein, *J. Biol. Chem.*, **201**, 45 (1952).
(5) H. H. Tallan, W. H. Stein and S. Moore, *J. Biol. Chem.*, **206**, 825 (1954).
(6) C. H. W. Hirs, S. Moore and W. H. Stein, *ibid.*, **195**, 669 (1952).
(7) W. H. Stein and S. Moore, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 179 (1949).
(8) G. Ehrensvärd, L. Reio, E. Saluste and R. Stjernholm, *J. Biol. Chem.*, **189**, 93 (1951).
(9) S. Åqvist, *Acta Chem. Scand.*, **5**, 1031, 1046, 1065 (1951).
(10) O. G. Lien, E. A. Peterson and D. M. Greenberg, *Anal. Chem.*, **24**, 920 (1952).
(11) J. S. Wall, *ibid.*, **25**, 950 (1953).
(12) M. V. Simpson and S. F. Velick, *J. Biol. Chem.*, **208**, 61 (1954).

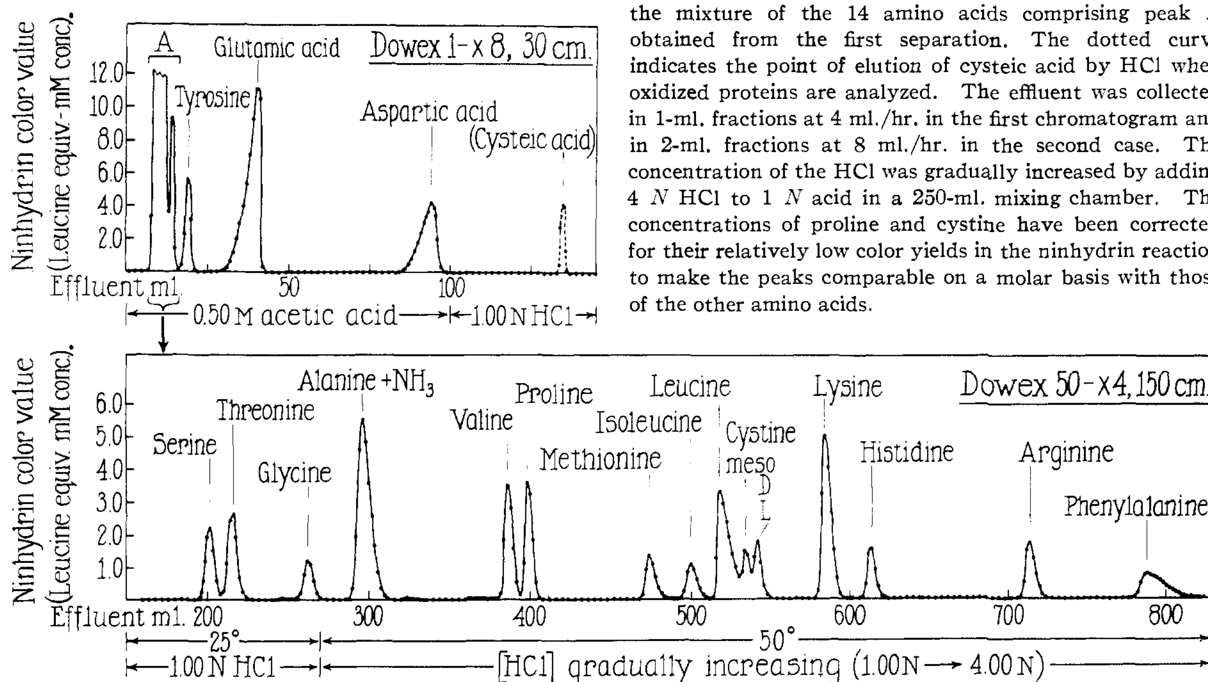

Fig. 1.—The elution of amino acids from Dowex **1-X8** with 0.5 N acetic acid at 25° and from Dowex 50-X4 with 1 to 4 N HCl at 25 and 50°. A hydrolysate from 70 mg. of bovine serum albumin (containing an additional 1.64 mg. of methionine) was chromatographed on columns 0.9 cm. in diameter. The sample added to the second column was the mixture of the 14 amino acids comprising peak A obtained from the first separation. The dotted curve indicates the point of elution of cysteic acid by HCl when oxidized proteins are analyzed. The effluent was collected in 1-ml. fractions at 4 ml./hr. in the first chromatogram and in 2-ml. fractions at 8 ml./hr. in the second case. The concentration of the HCl was gradually increased by adding 4 N HCl to 1 N acid in a 250-ml. mixing chamber. The concentrations of proline and cystine have been corrected for their relatively low color yields in the ninhydrin reaction to make the peaks comparable on a molar basis with those of the other amino acids.

50 columns.[1,2,6] The directions apply to chromatographic separations on columns 0.9 cm. in diameter with a load of 70 mg. of protein hydrolysate (100 mg. per sq. cm. cross-sectional area of the column). For larger loads, the cross-sectional area of each column, the volume of the effluent, and the size of the mixing chamber (see below) should be increased proportionately.[5]

Dowex **1-X8**.—The 200–400 mesh resin (Technical Service and Development, Dow Chemical Co., Midland, Mich.) in the chloride form, is suspended in two volumes of water and decanted after two minutes of settling, to remove any dense, white particles that settle rapidly. The bulk of the resin is screened through a 200-mesh sieve (74 μ openings) in the manner detailed for Dowex 50.[2] The through-200 mesh resin is suspended in water, allowed to settle for one hour, and the supernatant suspension is decanted to remove fine particles. This step is repeated until the supernatant fluid remains clear. The resin is converted to the acetate form by washing it on a büchner funnel with 3 M sodium acetate until the filtrate is chloride free (*cf.* Busch, *et al.*[13]). After thorough washing with water to remove all traces of sodium salts, the resin is washed with 0.5 M acetic acid and suspended in this solvent for the pouring of the columns. Several hold-up volumes of 0.5 N acetic acid are passed through the column before use.

Dowex **50-X4**.—The resin was prepared for chromatography as previously described.[2] The sodium salt was washed with 2 N HCl to remove all traces of ash, and the resin was equilibrated with 1 N HCl for the pouring of the 150-cm. columns in 30-cm. sections. Water jacketed chromatograph tubes were employed. The prepared column was washed well with the acid before the chromatogram was started. The variations in the chromatographic performance of different lots of Dowex 50-X4 have been discussed.[2] The resin used for the curve in Fig. 1 was a sample which had been adjusted by admixture with a small amount of an -X5 resin to give the standard performance defined for sodium Dowex 50-X4 columns.[2] In order to separate the components of the relatively simple mixture remaining after the use of the Dowex 1 column, it seems likely that most commercial samples of Dowex 50-X4 would be adequate, and Dowex 50-X8 columns might be applicable in some instances. A test run with a synthetic mixture of amino acids on the acid form of a given commercial batch of Dowex 50 would serve to show whether the desired degree of separation could be achieved without special adjustment of the resin. A newly poured column is required for each experiment.

Performance of the Chromatograms.—The acid hydrolysate from 70 mg. of bovine serum albumin was taken to dryness and transferred to the Dowex 1 column with 2 ml. of 0.5 N acetic acid. The effluent was collected in 1-ml. fractions on an automatic fraction collector at a rate of 4 ml./hr. (6 ml./hr. per sq. cm. cross-sectional area of the column). The chromatogram was operated at a room temperature of 25°. Aliquots of 0.05 ml. were pipetted[14] from the effluent fractions for analysis using 1 ml. of a strongly buffered ninhydrin reagent.[15] Crystalline aspartic and glutamic acids and tyrosine were obtained upon concentration of the appropriate pooled fractions.

The fractions corresponding to peak A in Fig. 1 were combined, including any crystals of cystine that may have separated, taken to dryness in a rotary evaporator[16] and dissolved in 2 ml. of 1 N HCl for addition to the Dowex 50 column. The effluent in this case was collected in 2-ml. fractions, at a rate of 8 ml./hr. After the emergence of glycine, the HCl concentration of the eluant was gradually increased by feeding 4 N HCl into a 250-ml. mixing chamber[2] containing the 1 N acid. The temperature of the water circulating through the jacket of the chromatograph tube was also raised at this point from 25 to 50°, the higher temperature serving to improve the separation of methionine from isoleucine.[1,17] For analysis by the ninhydrin method, 0.10-ml. aliquots of the effluent were pipetted. The samples were neutralized with the appropriate quantity of NaOH, if they corresponded to more than 0.1 ml. of 1.0 N HCl (all effluent fractions after the alanine peak in Fig. 1 were neutralized).

The pooled effluent fractions containing a given amino acid hydrochloride were concentrated to dryness in a rotary evaporator, transferred to a test-tube and taken to dryness under a filtered air stream on a steam-bath. To remove the last traces of HCl, the contents of the tube were transferred with 1 to 2 ml. of 0.5 N acetic acid to a 0.9 × 4-cm. bed of

(13) H. Busch, R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, **196**, 717 (1952).

(14) S. Moore and W. H. Stein, *ibid.*, **176**, 367 (1948).

(15) S. Moore and W. H. Stein, *ibid.*, in press.

(16) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

(17) H. Boström and S. Åqvist, *Acta Chem. Scand.*, **6**, 1557 (1952).

Dowex 1-X8 acetate, the effluent being collected from the beginning of the addition. The bed was washed with 0.5 *N* acetic acid until 0.05-ml. aliquots from successive 1-ml. portions of the effluent were ninhydrin negative. The first 3 ml. of effluent usually contained all of the amino acid. Thus freed of HCl and residual material from the resin, the neutral amino acids were obtained in crystalline form upon evaporation of the acetic acid and could be recrystallized from a suitable solvent.[6] The basic amino acids are most conveniently crystallized as their hydrochlorides,[6] sufficient HCl being added back for this purpose. In the case of alanine, a 0.9 × 3-cm. bed of Dowex 1 was initially converted to the free base form (*cf.*, Drèze, *et al.*[3]) by NaOH and washed thoroughly with freshly boiled (CO_2-free) water. When the mixture of alanine hydrochloride and ammonium chloride was transferred to the bed, the NH_3 was washed through with 2 to 3 ml. of water, and the amino acid was eluted with 0.5 *N* acetic acid. The alanine emerges in 5 ml., starting just before the break-through of acetic acid, as in the desalting of amino acid solutions on Dowex 2.[3]

Discussion

In the present experiments, the recoveries of glutamic acid and aspartic acid from Dowex 1-X8 were only 85 and 70%, respectively. When buffered columns of Amberlite IR-4B were used in the NH_4 acetate system,[6] the amino acids were recovered quantitatively. The reason for the losses of these two amino acids when chromatographed in dilute acetic acid on the quaternary base resin is not known. The results of the Dowex 50 chromatogram show that there is no carry-over of either of the amino acids into the second step of the procedure.

The recoveries from the Dowex 50-X4 column were quantitative, except for methionine, which presents a special case, since a portion of this amino acid may undergo oxidation to the sulfoxide during hydrolysis and during chromatography. The yield of methionine was 70% in the experiment given in Fig. 1. Since the protein itself is low in methionine (0.8%), an additional 1.64 mg. of methionine was added to the serum albumin before hydrolysis in order to provide a sufficient quantity of the amino acid to permit an accurate determination of the recovery. Traces of sulfoxide that might be formed during hydrolysis would emerge from Dowex 50-X4 near alanine. After oxidation of this mixture by performic acid, rechromatography would separate alanine from the sulfone, which moves ahead near the position of the glycine peak. Methionine that is gradually transformed to the sulfoxide during chromatography will not give rise to a peak on the effluent curve, but would be present (at a level undetectable by the ninhydrin method) in the region between the positions of emergence of alanine and methionine.

Tyrosine and cystine frequently crystallize in the effluent from the Dowex 1-X8 column prior to concentration of the solution. About 40% of the cystine present crystallized out in the experiment described in Fig. 1, but was added back to fraction A for chromatography on Dowex 50-X4. The double peak in the cystine position indicates the presence of the meso- and D- or L-forms of the amino acid formed as a result of partial racemization during acid hydrolysis.[18] The leucine–cystine separation can be improved by operation of the chromatogram at a temperature lower than 50°, either during the initial separation on the Dowex 50-X4 column or during rechromatography of material from the leucine–cystine zone. At 25° the two amino acids are widely separated, but the resolution of methionine and isoleucine is less satisfactory. If the cystine in the original protein has been oxidized by performic acid,[19,20] the resulting cysteic acid may be eluted from the Dowex 1-X8 column with 1*N* HCl (Fig. 1). The elution at the point of break-through of HCl obtained under these conditions provides little resolving power, and separation of cysteic acid from other strong organic acids possibly present in the sample would require the use of a less strongly acidic eluant.[19]

Tryptophan emerges at 32 ml. from a column of Dowex 1-X8 operated as described in Fig. 1. A longer column would be necessary to provide complete separation of tryptophan from glutamic acid, if detectable quantities of tryptophan were to survive the hydrolytic procedure.

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(18) C. H. W. Hirs, W. H. Stein and S. Moore, *J. Biol. Chem.*, in press.

(19) E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954).

(20) C. H. W. Hirs, *Federation Proc.*, **13**, 230 (1954).