CySS, 0.56; Ser, 0.97; Thr, 1.02; Gly, 1.03; Ala, 1.09; Pro, 0.93; Val, 0.99; Met, 1.21; Leu and Isoleu, 1.10; Phe, 10.3; Try, 1.54; Lys, 0.64; His, 1.62; Arg, 1.06. When the chromatograms are run in triplicate, the molar ratios are found to be reproducible to 0-10%.

When it is necessary to estimate the absolute amounts of the amino acids present in the mixture, two independent methods of calculation are available. The less accurate one (2-5%) involves a direct conversion of the optical density readings to micromoles by means of a set of effective millimolar coefficients; they have the values of 15.6/Fm where F represents the above set of factors. The greater inaccuracy of this procedure arises from differences in the absolute recovery of color from replicate chromatogams, these differences are not reflected in the molar ratios, however, since the variation seems to affect each amino acid to a comparable extent. The preferable method, therefore, combines the more accurate (2-3%) molar ratios with the total quantity of amino acids present in the mixture; the latter figure, in turn, can readily be deduced from the alkali uptake which accompanies the reaction of the amino acid mixture with FDNB. Thus, under the above-mentioned reaction conditions, the alkali uptake is found to be 77 ± 1% of the theoretical maximum (*i.e.*, 2 equivalents per NH_2 group, 1 equivalent for tyrosine-OH, and 0.25 equivalent for the imidazole ring of histidine), after due correction is made for the formation of dinitrophenol (0.44 μM of OH^- $ml.^{-1}$ $min.^{-1}$). The latter by-product does not interfere with the chromatography when the condensation with FDNB is carried out as described above in a solution approximately 0.01 *M* with respect to amino acids; for more dilute solutions, however, it is necessary to reduce the dinitrophenol concentration by sublimation (Mills, 1952) before application to the paper.

4. Comments

Heikens (1954) has shown that polymerization reactions may take place during dinitrophenylation. This observation indicates that care must be taken during the preparation of the DNP-derivatives of the amino acids. The preparation, purification and composition of the DNP-amino acids has been given by Rao (1954). The DNP-amino acids may also be separated on a silica-gel-celite column (Green, F. C., 1952). Zeile (1949) used the p-phenylazoanilinoformyl amino acids in place of the DNP-derivatives (*cf.*, Turba, 1948b; Schlögl, 1954).

CHAPTER VI

The Determination of Amino Acids by Column Chromatography on Ion Exchange Resins

By William G. Gordon, Ph.D.

INTRODUCTION

In 1951 Moore and Stein described a procedure for the chromatographic separation of mixtures of amino acids by elution analysis on columns of Dowex 50, a synthetic ion-exchange resin. In conjunction with a photometric ninhydrin method previously reported by these authors, whereby quantitative analysis of the effluent fractions may be carried out, the procedure permits accurate determination of the commonly occurring amino acids in acid hydrolyzates of proteins. The reliability of these techniques has now been amply demonstrated through successful use in many different laboratories. It is quite clear that these contributions to precise amino acid analysis are of great importance.

In this chapter primary emphasis will be placed on the experimental details of the ion-exchange method and its use in amino acid analysis of proteins. Only a few pertinent articles will be cited as illustrative of the earlier developments in column chromatography of amino acids. Reviews of the subject by Wieland (1943), Martin (1945), Tiselius (1947), Block (1949c) and Samuelson (1953) may be consulted for additional information.

SEPARATION OF BASIC AMINO ACIDS

Early attempts to apply ion-exchange reactions to the separation of mixtures of amino acids showed that group fractionations, depending primarily on the net charge and sign of charge of the amino acids, were feasible. For example, the experiments of Whitehorn (1923) demonstrated that only the basic amino acids, arginine, lysine and histidine, were adsorbed from neutral solution by Permutit (a synthetic sodium aluminum silicate).

Not only were group separations of basic amino acids possible by

these methods, but also, as demonstrated by Turba (1941), separations of the individual diamino acids, Turba used Filtrol, Neutrol and Floridin XXF, ion-exchange materials of the fuller's earth type, in his studies. When a mixture of monoamino and diamino acids was put on a column of Floridin XXF and eluted with water, arginine and lysine remained in the column, while histidine and monoamino acids emerged. Histidine was separated by adsorption on Filtrol-Neutrol and subsequent elution with a sulfuric acid-pyridine mixture. Arginine and lysine were eluted from the original column by sulfuric acid-pyridine; the solvent was removed, the mixture taken up in M/6 KH_2PO_4, and the solution run through a column of Filtrol-Neutrol. Arginine was retained while lysine was recovered from the eluate. The arginine was finally eluted with sulfuric acid-pyridine (*cf.*, Block and Bolling, 1951, p. 36 *et seq.*).

Other ion-exchange materials, such as metallic oxides (TiO_2, Al_2O_3, silica gel) and special synthetic resins, have been used also in these separations. The demonstration by Adams and Holmes (1935) that synthetic resins are capable of exchanging ions, was of general importance in that it became possible to produce materials suited particularly to separations of ions of specific kinds. The first applications of synthetic resins to amino acid separations were made by Block (1942) and independently by Freudenberg (1942) in the same year. Block found that the basic amino acids could be separated from other amino acids and, in later work, that large-scale fractionations of protein hydrolyzates could be made in this way (*cf.*, Block, 1949).

SEPARATION OF DICARBOXYLIC AMINO ACIDS

When Al_2O_3 is treated with dilute acid, it acquires a positive charge and it may then be used as an ion-exchange material to adsorb aspartic and glutamic acids from mixtures of amino acids. This observation by Wieland (1942) was developed by him and others into a method for the separation of the dicarboxylic acids from protein hydrolyzates and for the separation of the two acids from each other (*cf.*, Block and Bolling, 1951, Chapter VI). Thus, Darling (1945) used a column of Al_2O_3, previously treated with *N* HCl and washed with water, to adsorb aspartic and glutamic acids from a neutralized mixture of amino acids. The other amino acids were washed out with water. Glutamic acid was then eluted with dilute acetic acid, aspartic acid being retained on the column. Dilute KOH served to recover the aspartic acid.

The first use of a synthetic ion-exchange resin for the quantitative separation of dicarboxylic acids from a protein hydrolyzate was that of Cannan (1944). A hydrochloric acid hydrolyzate was treated with enough Amberlite IR-4 (a synthetic polyamine-formaldehyde resin in free-base form) to raise the pH to 7. The dicarboxylic acids could be adsorbed quantitatively by a number of such treatments. The batches of resin were washed with water and the aspartic and glutamic acids recovered together by elution with excess HCl.

Amberlite IR-4 was also used by Consden (1948) to adsorb the dicarboxylic amino acids and, further, to separate them effectively. In this method a column of the resin maintained at pH 3 to 4 retained the dicarboxylic acids in a protein hydrolyzate. After the column was washed with water, glutamic and aspartic acids together were eluted with *N* HCl. Excess HCl was removed and the dicarboxylic acids were transferred to a second column of the resin equilibrated at pH 2.5. At this pH glutamic acid was eluted first, followed by aspartic acid.

SEPARATION OF NEUTRAL AMINO ACIDS

The prime importance of charge in the exchange separations of amino acids described in the preceding paragraphs is obvious. However, other phenomena, such as true adsorption and partition, are also operative in ion-exchange column chromatography. Indeed adsorption chromatography and partition chromatography alone have been used for separations of neutral amino acids.

1. True Adsorption

During the course of a broad investigation of adsorption analysis begun in 1941, Tiselius demonstrated that simple mixtures of neutral amino acids could be separated by virtue of different affinities of the components for active carbon. Important new methods of chromatographic analysis were introduced as the work progressed (*cf.*, Tiselius, 1947; Block and Bolling, 1951, p. 400 *et seq.*). Three different methods used by Tiselius, *elution analysis*, *frontal analysis* and *displacement analysis*, will be described in general terms.

In *elution analysis*, the normal procedure in column chromatography, a small volume of the solution to be analyzed is forced into the top of a column of washed adsorbent. Pure solvent, in large quantities, is then passed through the column. The components of the mixture move down the column in separate zones. Pure solvent emerges first, followed by a solution of pure, least strongly adsorbed

component, then pure solvent, another component, etc. To remove most strongly adsorbed components quantitatively, it is often more efficient to change the composition of the solvent during the course of elution.

In *frontal analysis*, the solution to be analyzed is passed continuously through the column. The different components are retarded relative to the solvent in their passage through the adsorbent, each component exhibiting a specific retardation dependent on the extent of adsorption. If the original solution contains *A* and *B*, both components are adsorbed and move through the column as sharp, separate fronts as the adsorbent becomes saturated with respect to each component. If *B* is more strongly adsorbed than *A*, then pure solvent emerges first, followed by a sharp step in concentration as *A* emerges, and finally, by a second step as *B* emerges together with *A*. The composition of the effluent is then identical with that of the original solution.

In *displacement analysis*, as in elution analysis, a small volume of the solution to be analyzed is introduced into the column but instead of pure solvent as developer, a solution of a substance ("developer") more strongly adsorbed than any of the components is used. Actually a frontal analysis of the "developer" occurs behind the components of the original solution and the latter are pushed through the column just ahead of the advancing front of the "developer." The displaced components gradually form sharply separated zones, each containing a single component. Pure solvent emerges first, followed by the components, well separated but in close proximity to each other, and finally by the front of the "developer." None of the "developer" appears in the component solutions which precede it.

The application of these principles of adsorption analysis to ion-exchange chromatography has been discussed by Samuelson (1953).

2. Partition

Partition chromatography was introduced by Martin and Synge in 1941 (*cf.*, Block and Bolling, 1951, p. 405 *et seq.*) as a means of separating acetylamino acids. In place of the normal solid adsorbent in column chromatography, an inert powder (precipitated silica) was used as a mechanical support for a stationary liquid phase (water). The column was run with a second liquid phase (such as chloroform-butanol) immiscible with the first. In this system the physical property which made separations possible was partition between the two liquid phases, rather than adsorption on the solid phase. In fact, free amino acids could not be well separated in this manner because of adsorption on the silica gel. For a particular solvent mixture, each acetamino acid moved down the column at a definite rate and could be collected as it emerged. The method was later developed by Gordon (1943) into a standard procedure for the determination of phenylalanine, leucine plus isoleucine, valine, alanine, proline, tyrosine and methionine in hydrolyzates from 25 mg. of protein.

Amino acids, without prior acetylation, were also separable by partition chromatography when raw potato starch was used as mechanical support for the water phase and 1-butanol was the immiscible solvent (Synge, 1944). A quantitative analytical method based on these observations was worked out by Stein (1949).

GENERAL METHODS

The principles of exchange, true adsorption and partition become interrelated in the practice of ion-exchange chromatography. In combination they are responsible for the high resolving power of the method. Even complex mixtures of closely related compounds such as are in protein hydrolyzates can thus be resolved completely. Different procedures have been elaborated for isolation and for quantitative analysis.

1. Isolation

Displacement Analysis: Partridge, in a series of papers, studied displacement chromatography on ion-exchange resins as a means of separating amino acids. This work is summarized in the systematic method of Partridge (1952a; 1952b) (*cf.*, Davies, 1950; Hagdahl, 1954ab; Kunin, 1951). Three resins were used: the cation exchangers, sulfonated cross-linked polystyrene and Zeo-Karb 215, and the strongly basic anion exchanger, Dowex 2. In a preliminary step the amino acid hydrochlorides from a yeast protein hydrolyzate were treated with active charcoal to remove tyrosine and phenylalanine which, if allowed to remain, behaved irregularly later in the procedure. The aromatic amino acids were recovered by elution with aqueous phenol-acetic acid and separated by fractional crystallization. The charcoal-treated filtrate was then fractionated as follows:

Primary Separation: First Stage. The mixture was adsorbed on a column of sulfonated polystyrene resin and displaced with dilute NaOH. Fractions of the eluate were collected by an automatic device. The early fractions contained the acidic and neutral amino acids and

the later ones, the basic amino acids. Lysine and arginine were well resolved and required no further purification. The crude histidine fraction was adsorbed on Dowex 2 and displaced with dilute HCl. Some additional lysine was displaced first and later, pure fractions of histidine were obtained.

Primary Separation: Second Stage. The early fractions from the first stage were combined. The mixture was adsorbed on Zeo-Karb 215. The displacement developer was dilute NH_4OH. The effluent showed five fairly well defined bands of the following composition:

I. Pure aspartic acid.
II. Glutamic acid, serine, threonine.
III. Glycine, alanine.
IV. Valine, proline.
V. Leucine, isoleucine, methionine.

Band I required no further purification but there was minor overlapping in some of the other bands. Each of the mixed bands was passed through a column of Dowex 2, and displaced with dilute HCl. This effected final purification of each amino acid except that the pairs, leucine-isoleucine and serine-threonine, were not resolved. However, serine-threonine could be separated by displacing the mixture from a column of sulfonated polystyrene resin, using NaOH dissolved in aqueous acetone as displacement developer.

Cystine was present only in traces in the yeast hydrolyzate. In another experiment with egg albumin it was found that most of the cystine occurred in Band V, while some was present in the basic amino acid fraction. Cystine was readily isolated from these by simple crystallization.

This system was developed as a preparative method for the isolation of amino acids from quantities of protein ranging from 10 to 250 grams. Displacement analysis has an advantage over elution analysis in its higher capacity for a resin column of given size. Elution analysis, however, because of its higher resolving power, is more suitable for quantitative analysis and for isolation work on a small scale.

Elution Analysis: Hirs (1952, 1954a) developed two procedures for the isolation of small amounts of amino acids by ion-exchange chromatography and elution analysis. The first method (1952) using volatile buffers as eluting agents was applied to an acid hydrolyzate of 2.5 g. of bovine serum albumin. For complete separation of amino acids four columns of resin were required: Dowex 50 (NH_4^+) for the basic amino acids, Amberlite IR-4B (CH_3COO^-) for the acidic amino acids, and two columns of Dowex 50 (NH_4^+) for the neutral amino acids. Elution was effected with ammonium formate or acetate buffers in the range, pH 3 to 7.

Each of the well-resolved amino acids was isolated by removal of the buffer by sublimation and subsequent crystallization. All the amino acids (except methionine) were obtained in analytically pure form in an average yield of 66%.

The second method (Hirs, 1954a) is somewhat simpler, involving fewer steps and the use of a volatile acid as the eluting agent. An acid hydrolyzate of 70 mg. of bovine serum albumin was used. The amino acids were first chromatographed on Dowex 1-X8 acetate (a strongly basic anion exchanger containing quaternary ammonium groups attached to a styrene-divinylbenzene copolymer; X8 means that the resin contains 8% divinylbenzene and is a measure of the amount of cross-linking). In the chromatogram dilute acetic acid eluted Fraction *A* (containing most of the amino acids) then tyrosine, glutamic acid and aspartic acid, each well separated. Fraction *A* was taken to dryness to remove the acetic acid. The residue was dissolved in *N* HCl and rechromatographed on Dowex 50-X4 (H^+). Elution was begun with *N* HCl at 25° C. Serine, threonine, and glycine were obtained in well resolved peaks. The temperature was raised to 50° C. and, at the same time, the composition of eluting agent was changed gradually from 1 *N* to 4 *N* HCl by a mixing device.* Pure amino acid peaks were collected in the following order: alanine (plus ammonia), valine, proline, methionine, isoleucine, leucine, cystine, lysine, histidine, arginine and phenylalanine. Each amino acid was isolated by crystallization. Recoveries were quantitative with the following exceptions. Only 85% of the glutamic acid and 70% of the aspartic acid were recovered from the Dowex 1 column. Methionine presented a special case in this work and was not recovered quantitatively.

2. Quantitative Analysis

Starch Column Chromatography (Stein, 1949). As mentioned previously, partition chromatography on potato starch columns has been used successfully in amino acid analysis by Stein (1949). For quan-

* This consists of a 250-ml. chamber (a modified, ground joint wash bottle), equipped with magnetic stirrer, and filled with 1 *N* acid; 4 *N* acid is then fed in gradually. Details are given by Moore (1954a).

titative analysis of the separated amino acids in the effluent fractions, an accurate spectrophotometric ninhydrin method was employed (Moore, 1948). Three chromatograms on starch columns were required for complete separation and determination of all amino acids and ammonia in a protein hydrolyzate. The starch columns were prepared with moist potato starch suspended in 1-butanol and then equilibrated with the solvent to be used in the run.

For the first chromatogram an aliquot of the amino acid hydrochlorides equivalent to about 2.5 mg. of protein, was added to the column, and the column was developed with *n*-butyl alcohol:*n*-propyl alcohol: 0.1 *N* HCl = 1:2:1 v/v. Fractions of the effluent were collected mechanically. The amino acids emerged in the following order: leucine plus isoleucine, phenylalanine, valine plus tyrosine plus methionine, proline, alanine plus glutamic acid, threonine, and aspartic acid. Only the proline, threonine and aspartic acid peaks were sharply resolved and suitable for quantitative analysis. The solvent was then changed to *n*-propyl alcohol:0.5 *N* HCl = 2:1 v/v which separated the remaining amino acids into pure peaks: serine, glycine, ammonia, arginine, lysine, histidine and cystine. The ten pure fractions from this chromatogram were analyzed.

A second chromatogram with another aliquot of the hydrolyzate developed with *n*-butanol:benzyl alcohol:water = 100:100:28.8 v/v as solvent yielded well resolved peaks of phenylalanine, leucine, isoleucine, methionine, tyrosine and valine. These were the first amino acid peaks to emerge and were suitable for analysis.

The final chromatogram was run with a third aliquot and *tert*-butyl alcohol:*sec*-butyl alcohol:0.1 *N* HCl = 2:1:1 v/v as solvent. A fore fraction containing the leucines, phenylalanine, valine, methionine and tyrosine was eluted prior to the emergence of proline. Pure fractions of glutamic acid and alanine were then collected and analyzed.

Each chromatogram was run with about 2.5 mg. of protein. β-Lactoglobulin and bovine serum albumin were analyzed by this method and excellent total nitrogen and weight recoveries of each protein in terms of known amino acids and ammonia were reported. The accuracy of the analytical method may best be judged by recoveries of 100 ± 3% for the individual components of synthetic mixtures of amino acids. Although the method has been in general use since its publication, the more convenient ion-exchange procedure of Moore (1951) superseded it. The resin columns employed in the latter method have higher resolving power than starch columns, and are not adversely affected by the presence of inorganic salts. The newer method is also faster.

Ion Exchange Chromatography (Moore, 1951)

(a) *Principle.* A mixture of amino acids (3 to 6 mg.) is added to a 0.9 × 100 cm. column of Dowex 50-X8 (Na^+).* The amino acids are eluted by a series of buffers of progressively increasing pH, from 3.4 to 11, at different temperatures. The effluent is collected by means of an automatic fraction cutter in 1-ml. portions in optically calibrated tubes. The amino acids emerge in a definite sequence, well separated from each other. Ninhydrin reagent is added to each tube for spectrophotometric determination of amino acid concentration. Summation of the quantities of amino acid found in the appropriate group of tubes gives the yield of each amino acid. Quantitative recoveries (100 ± 3% on the average) are obtained for all but the basic amino acids. These and ammonia are determined quantitatively with equal accuracy in a separate experiment on a 0.9 x 15 cm. column of the resin with buffers ranging from pH 5 to 6.8 at 25° C.

(b) *Equipment and Reagents.* (1) *Fraction Collector:* An automatic machine designed to collect successive fractions of effluent of known volume from the chromatograph column is required. A number of such machines are available commercially.† One, accommodating 200 tubes, and based on the original design of Stein (1948) is manufactured by the Technicon Company, Chauncey, New York. Also available from this source are convenient 50-tube aluminum racks for handling the tubes used both in the collector and spectrophotometer.

(2) *Spectrophotometer:* An instrument such as the Coleman junior or the Beckman Model B spectrophotometer, wherein direct readings of optical density in 18 x 150 mm. test tubes may be made, is needed.

(3) *Calibration of Test Tubes:* A matched set of at least 400 test tubes (18 x 150 mm., without lips) should be calibrated. Prepare a solution of methyl red in 0.03 *N* HCl of such strength as to give a reading at 525 mμ of 0.6 to 0.7 on the optical density scale against a water blank.** Add 5 to 10 ml. of the solution to each tube, and select only those tubes which read within 0.005 unit of the same optical density value. Mark permanently the side of the tube facing the

---

* Mention of products or of apparatus in this chapter does not imply endorsement or recommendation of the Department of Agriculture over similar products or apparatus not mentioned.

† Literature references concerning the design of various types of fraction collectors may be found in an article by Schroeder (1954).

** Instead of the methyl red solution, the more stable solution of 0.12 *M* $CoCl_2 \cdot H_2O$ in 0.25 *N* HCl at a wave length of 515 mμ is preferable for these calibrations (Gordon, unpublished).

light source and align the tube in the same way each time it is read. Number the selected tubes with a glass marking tool.

Aluminum caps are convenient covers for the tubes during the course of analysis.

(4) *Boiling Water Bath:* This bath, for ninhydrin color development, should be large enough to hold a rack of 50 tubes immersed in vigorously boiling water to a depth of about 5 cm.

(5) *Constant Temperature, Circulating Water Bath:* This is required to maintain the temperature of the 100 cm. column at 37.5, 50 and 75° ± 0.5° C.

(6) *Pipetting Machine:* Although such a device is not essential, it saves much time in dispensing reagents. Suitable instruments are sold by the Baltimore Biological Laboratories, Inc., Baltimore, Md., and by the National Instrument Co., Baltimore, Md.

(7) *Buffers:* These may be prepared in quantity, adjusted if necessary with a little *N* HCl or *N* NaOH, and stored in the cold with thymol.

(a) Citrate buffer, 0.2 *M*, for ninhydrin solution. Dissolve 21.008 g. of citric acid ($C_6H_8O_7 \cdot H_2O$) in 200 ml. of *N* NaOH and dilute to 500 ml. with H_2O. The pH of the buffer when diluted with an equal volume of H_2O should be 5.0 ± 0.1.

(b) Buffer, pH 3.42 ± 0.01. To 500 ml. of the above citrate buffer (0.2 *M*) add 110 ml. of 1.0 *N* HCl and 390 ml. of H_2O. Prepare this buffer in 10 l. quantities. Before use, add 0.5 ml. of thiodiglycol and 1 ml. of BRIJ 35 solution per 100 ml. of buffer.*

(c) Buffer, pH 4.25 ± 0.05. To 500 ml. of citrate buffer (0.2 *M*) add 50 ml. of N HCl and 450 ml. of H_2O. Before use, add 0.5 ml. of thiodiglycol, 1 ml. of benzyl alcohol,† and 1 ml. of BRIJ solution per 100 ml. of buffer.

(d) Buffer, pH 5.0 ± 0.1. To 500 ml. of citrate buffer (0.2 *M*) add 500 ml. of H_2O. Before use, add 0.1 g. of disodium versenate,** 1.5 ml. of benzyl alcohol, and 1 ml. of BRIJ solution per 100 ml. of buffer.

(e) Buffer, pH 6.8 ± 0.03. To 500 ml. of 0.1 *M* Na_2HPO_4 add 450 ml. of 0.1 *M* $NaH_2PO_4 \cdot H_2O$. Before use, add versenate, benzyl alcohol and BRIJ as for preceding buffer.

(f) Buffer, pH 6.5 ± 0.05. Dissolve 42 g. of citric acid ($C_6H_8O_7 \cdot H_2O$) in 580 ml. of 1.0 *N* NaOH and dilute to 1 liter with H_2O. Before use add versenate, benzyl alcohol and BRIJ as for preceding buffer.

(8) *Ninhydrin:* The product sold by Dougherty Chemicals, Richmond Hill, New York, is sufficiently pure without recrystallization.

(9) *Methyl Cellosolve:* The reagent should be clear when mixed with an equal volume of water, and peroxide-free (test with 10% aqueous KI). Hamilton (1954) suggests that storage in the presence of zinc-copper couples will keep the reagent peroxide-free.

(10) *Ninhydrin Solution:* Dissolve 0.80 g. of reagent $SnCl_2 \cdot H_2O$ in 500 ml. of citrate buffer (0.2 *M*). Dissolve 20 g. of ninhydrin in 500 ml. of methyl cellosolve. Mix, and preserve the mixture under nitrogen. For fewer analyses, and especially if a pipetting machine is not available, it is also satisfactory to prepare only the minimum volume of ninhydrin solution required for daily use and to use a burette to measure out the solution for color development; in this case storage under nitrogen is not necessary.

(11) *Diluent Solution:* 1-propanol:water = 1:1 v/v.

(12) *Resin:* Dowex 50-X8, minus-400 mesh grade, may be obtained from Technical Service and Development, The Dow Chemical Company, Midland, Michigan. One pound of the resin is sufficient for the preparation of several 100 cm. columns.

(13) *Chromatograph Columns:* Zechmeister-Cholnoky type, with ground joints and coarse sintered glass plates; glass tubing 0.9 cm. inner diameter, 1.5 to 2 mm. wall thickness; length of tubes: for 100 cm. resin column, 115 cm., and for 15 cm. resin column, 25 cm.

The long tubes are jacketed to permit temperature control by circulation of water from the constant temperature bath.* The short tubes are operated at room temperature.

(c) *Preparation of Resin Columns*

Wash one pound of Dowex 50-X8 (H^+) with 4 to 8 liters of 4 *N* HCl on a Buchner funnel with gentle suction. The final washes should be almost colorless. Wash twice with distilled water and

---

* Thiodiglycol is added as an antioxidant to prevent losses of methionine. It may be obtained by the redistillation under reduced pressure of Kromfax solvent (Carbide and Carbon Chemicals Corp.) or of technical 2,2'- thiodiethanol (Eastman). BRIJ 35 (Atlas Powder Co., Wilmington, Del.) is a neutral, stable detergent, which permits faster flow during elution. Dissolve 50 g. of BRIJ 35 in 100 ml. of H_2O for the stock solution.

† Benzyl alcohol improves the resolution of tyrosine and phenylalanine.

** Analytical grade disodium versenate (Versenes Inc., Framingham, Mass.) containing negligible amounts of ninhydrin-positive impurities should be used. If versenate is not included in the buffers, low recoveries may be obtained after continued use of the 15 cm. columns.

* A convenient rubber centrifugal pump is manufactured by Schaar and Co., Chicago, Ill.

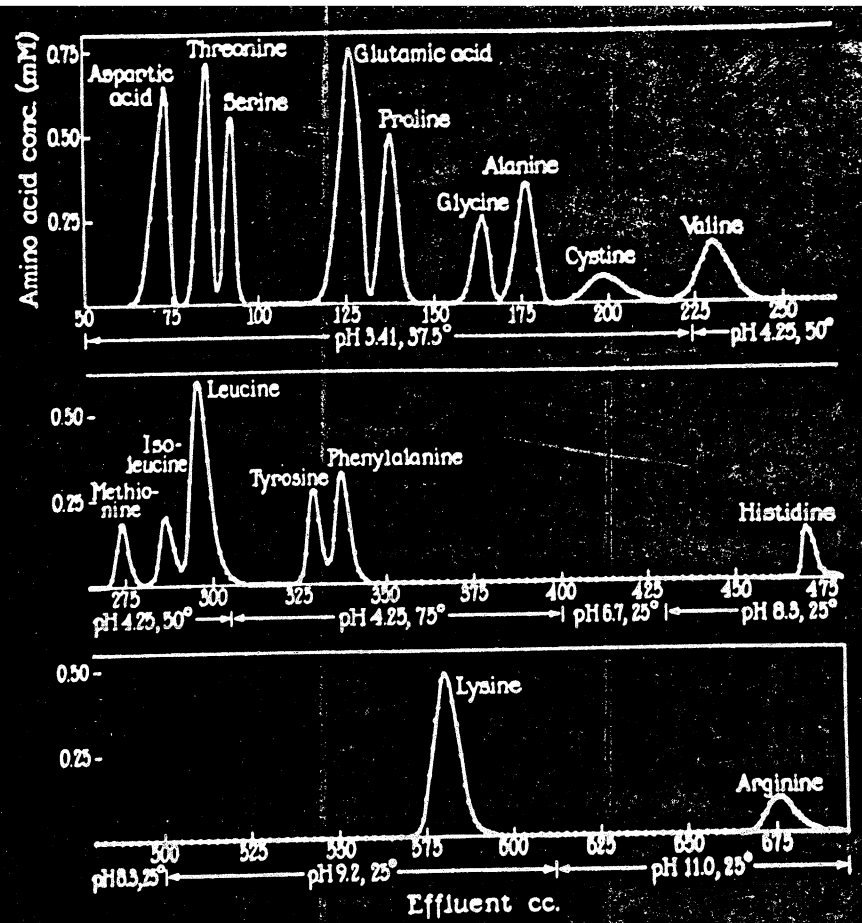

FIGURE 38. Separation of amino acids from a synthetic mixture simulating the composition of a protein hydrolyzate. The column of Dowex-50, 0.9 x 100 cm., was operated in the sodium form, with buffers of the pH and temperature indicated as eluants. A sample of about 6 mg. of amino acids was chromatographed. The position of an amino acid peak is reproducible, on the average, to better than 5%. (From Moore, 1951.)

then with 2 *N* NaOH until the filtrate is alkaline. Suspend the resin (Na^+) in about 3 times its volume of *N* NaOH and heat on a steam bath for about 3 hours with occasional shaking. Allow to settle for about ½ to 1 hour, decant the supernatant liquid, and add fresh, hot *N* NaOH. Repeat hot alkali treatment 5 times in all. The final washes are almost clear. Filter the resin, wash free of alkali and screen the wet resin through a 200 mesh sieve (74 μ openings) with the aid of a strong jet of water. Discard the large particles. Filter off the "through-200 mesh" resin and store as the moist sodium salt. The currently produced resin (1955) will give at least 50% yields of "through-200 mesh" resin. Moore (1954a) states that "through-200 mesh" resin gives elution curves superior to that shown in Figure 38 which was obtained with "through-120 mesh" resin. With the finer resin the leucine-isoleucine and tyrosine-phenylalanine separations are complete, and the cystine peak is sharper.

To prepare a column for use, wash some of the resin on a filter with a small amount of the pH 3.4 buffer (without detergent). Suspend the washed resin in about twice its volume of buffer. Allow to settle and remove bubbles by gentle stirring. Pour the slurry into the chromatograph tube in 5 portions of about equal volume (50 ml.), using a funnel with a bent, constricted tip to direct the flow of slurry against the side of the tube. Allow the first portion of resin to settle under air pressure of 10 to 15 cm. of mercury until there is no further drop in the height of the surface of the resin. Maintain the pressure until the liquid surface falls to within about 10 cm. of the resin surface. Then add a second portion of the slurry as before. Continue the additions of resin until a column of packed resin, 100 cm. in height, is obtained. If the top surface of the resin is irregular, stir it with a little supernatant buffer and allow it to settle again.

Prepare the 15 cm. column similarly in one or two sections.

Attach a separatory funnel (about 300 ml. capacity) as a reservoir for appropriate buffer to the column with a length of plastic tubing so that the funnel may function as a leveling bulb. Run about 100 ml. of buffer through the column. It is then ready for use.

(d) *Procedure for Photometric Ninhydrin Method* (Moore, 1948, 1951)

To 1 ml. of amino acid solution at about pH 5.0, add 2 ml. of ninhydrin solution. Cap the tube and mix by shaking. Heat in *vigorously boiling* water bath for 20 minutes. Allow to cool for a few minutes and add 5 ml. of diluent solution. Shake well for 1 minute. Read in the spectrophotometer beginning at about 15 minutes after removal from the bath, using 440 mμ for proline and hydroxyproline and 570 mμ for all other amino acids. Read reagent blanks against the diluent. Select average reagent blank, set instrument to zero on this blank, and read unknown. The reagent blank reading should be about 0.15 to 0.20 on the optical density scale. Analyses should be run in groups of not more than 50 tubes to permit readings

to be completed within 1 hour after the heating period; during this time readings remain essentially constant.

When optical density values at 8.0 ml. total volume are too high for accurate reading, further additions of one, two or three 5 ml. portions of diluent solution are made, the average reagent blank is diluted likewise, and readings are taken at 13, 18 or 23 ml. If readings at 23 ml. are still too high, aliquots of unknown and blank are pipetted into other calibrated tubes and further dilution is made.

Leucine Calibration Curve: On a molar basis the amino acids do not give the same color yield in the ninhydrin reaction. However, only one calibration curve is required because accurate color yields relative to leucine have been established. Relative to the leucine color value at 570 mμ taken as 100, the color yields per mole for other amino acids are: Hydroxyproline 3; aspartic acid 93; threonine 92; serine 97; glutamic acid 105; proline 5; glycine 101; alanine 99; cystine 108; valine 100; methionine 100; isoleucine 100; tyrosine 91; phenylalanine 91; tryptophan *ca.* 72; histidine 104; lysine 112; ammonia *ca.* 98; and arginine 100. Spectrophotometer readings taken at 440 mμ for proline (and for hydroxyproline, if present) are first converted to "micromoles leucine" using the leucine calibration curve at 570 mμ employed for the other amino acids; these values are then multiplied by 3.7 to give micromoles of proline and by 7.2 to give micromoles of hydroxyproline.

Prepare a calibration curve, plotting micromoles of leucine against optical density, with a sample of leucine of established purity. Use aqueous solutions of the amino acid at several different levels of concentration, within the range 0.05-0.20 micromoles of leucine per 1 ml. aliquot. This is a convenient range for ninhydrin color development by the above procedure in a total volume of 8 ml. Once the standard curve has been confirmed by replicate experiments, a standard table relating optical density to micromoles of leucine may be prepared and from these figures further tables may be calculated at total volumes of 13, 18 and 23 ml. for higher concentrations of amino acid.

(e) *Operation of 100 Cm. Columns*

With the prepared column in place above the fraction collector,* begin circulating water at 37.5° C. through the jacket about an hour before the start of a run. Heat to boiling about 300 ml. of buffer of pH 3.42 to free it of dissolved air, pour the warm buffer into the separatory funnel, and cover the solution with a thin layer of mineral oil. Add the sample of amino acid solution to the column with a 1 or 2 ml. pipette with bent tip so as not to disturb the surface of the resin. When the sample is drained into the resin, wash down in the same way with three successive 0.3 ml. portions of buffer, and finally layer about 2 ml. of the buffer above the surface of the resin. Do not allow the surface of the resin to dry out during these operations. The pH of the amino acid solution should be more acid than that of the buffered column, about 2.5 to 3.0 being optimal. Usually, a charge of about 5 mg. of total amino acid mixture is introduced for a chromatogram but several times this quantity is permissible and is recommended for more accurate determination of amino acids present in small concentration.

Connect the buffer reservoir and adjust the flow rate of solvent through the column to about 4 ml. per hour by adjusting the height of the separatory funnel or by using slight air pressure (*cf.*, Mueller, 1949). Collect the effluent in 1 ml. fractions.

Figure 38 shows that both buffer and temperature are changed at the beginning of the valine peak (about 225 ml.). Before this point is reached, it will have been possible to develop and analyze the ninhydrin color of tubes 100-150. Note the position of the yellowish-red proline peak. For example, tube 135 may have contained the maximal amount of proline. Multiply 135 by the factor 1.63 to give 220 ml. Use this figure as a better estimate of the start of the valine peak. At this point, change the setting of the temperature regulator in the water bath to 50° C. Replace the buffer pH 3.42 in the reservoir with boiled buffer of pH 4.25. Allow the surface of the 3.42 buffer in the column to fall to about 1 mm. above the resin surface, then with bent pipette add about 2 ml. of 4.25 buffer, and connect the reservoir of fresh buffer. As the temperature of the column rises, maintain the desired flow rate by lowering the pressure. Change the temperature to 75° C. when another 75 ml. have been collected (at about 295 ml.), and again adjust the flow rate. Usually, the chromatogram is stopped at about 385 ml. because the basic amino acids cannot be recovered quantitatively. Allow the column to cool to room temperature before closing the stop-cock of the separatory funnel.

If it is desired to complete the chromatogram as shown in Figure 38, consult the original paper of Moore (1951) for preparation of buffers and procedure. Two-dimensional paper chromatograms should be

* If the collector is of the Technicon type, with the tubes housed in an enclosed chamber, it is well to circulate a slow stream of ammonia-free air through the chamber; shallow dishes containing citric acid solution may also be used on top of the rack to minimize absorption of ammonia by the acidic eluent.

carried out to establish the presence of all the components of an unknown mixture. Because of the limited resolving power of the 15 cm. column, it cannot be relied upon to differentiate ornithine from lysine, hydroxylysine from histidine, or glucosamine from tryptophan.

At the end of the chromatogram, clean the column by running through about 100 ml. of 0.2 *N* NaOH containing BRIJ. Then run through 100 ml. of pH 3.42 buffer and the column is ready for use.

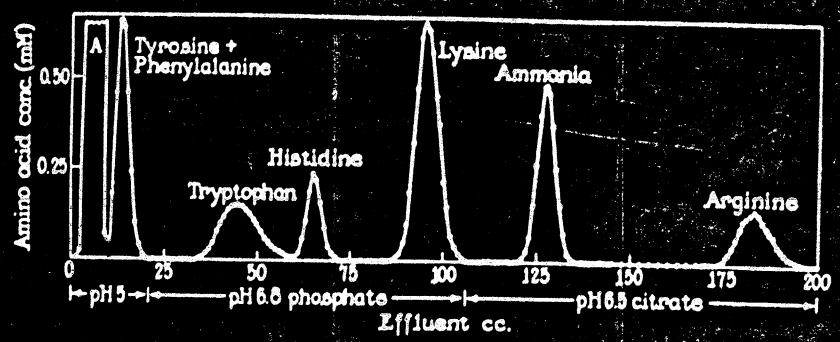

FIGURE 39. Separation of the basic amino acids on a column of Dowex-50, 0.9 x 15 cm. The column was operated in the sodium form at 25° C. with the buffers indicated. A sample of about 6 mg. of amino acids as in Figure 38 was employed. The large peak, *A*, comprises all the amino acids emerging before tyrosine in Figure 38. (From Moore, 1951.)

If the surface of the column becomes clogged at any time, remove the top cm. of resin and replace it with fresh resin. Never allow the resin to dry out.

(f) *Operation of 15 Cm. Columns*

Run the chromatogram at room temperature and as shown in Figure 39. Heating of the buffers is not required. Before use, run about 25 ml. of pH 5 buffer through the column. Add the load of amino acids in 1.0 or 2.0 ml. of solution of about pH 4. For the desired flow rate it may be necessary to lower the buffer reservoir and to operate it at negative pressure. Make the changes of buffer as indicated in Figure 39. If histidine is not well separated from lysine, adjust the pH of the 6.8 phosphate buffer to 6.85 or 6.90. Clean the column before reuse with about 25 ml. of 0.2 *N* NaOH plus BRIJ.

(g) *Analysis of Collected Fractions*

Develop the ninhydrin color in 50 tubes at a time. First adjust the pH of each fraction to about pH 5 with one or two drops of HCl or NaOH as required. The following figures give the approximate amounts required to adjust 1 ml. of buffer to pH 5: buffer 3.42, 0.1 ml. of *N* NaOH; buffer 4.25, 0.05 ml. of *N* NaOH; buffer 5.0, none; buffer 6.8, 0.05 ml. of 0.5 *N* HCl; buffer 6.5, 0.05 ml. of 2 *N* HCl. Determine the proper amount and strength of acid or alkali to be added by titrating aliquots of the different buffers before running the chromatogram. By calibrating the size of drops from a slowly dripping burette and by adjusting the strength of acid or alkali, establish the required number of drops for 1 ml. of each buffer.*

The hold-up volume of the 0.9 x 100 cm. columns is about 22 ml., but the more acidic buffers have a significant retardation volume. Thus, if the change of buffer to 4.25 is made at tube number 220, buffer 4.25 will not emerge from the column until tube number 255 (35 ml. later). Therefore, for the pH adjustment, change from 2 drops of alkali to 1 drop at tube 255. For the 15 cm. columns, make the changes in adjustment of pH, 4 ml. after the change of buffer.

Add ninhydrin and develop color as described. In making the optical density readings it is important to choose the proper blank against which amino acid concentration is read. In the aspartic acid-proline region (Figure 38) choose a representative blank from the valleys in the curve. For the glycine and alanine peaks, select a blank after the proline peak. The curve may or may not return to

---

* The development of a modified ninhydrin reagent with greatly increased buffering capacity makes possible analysis of the effluent fractions without adjustment of pH. If the modified reagent is used, the detailed procedure in the original paper (Moore, 1954b) should be followed. It will be noted that there are important points of difference in the procedure with the newer reagent: use of 1 ml. of reagent, 15 minute heating period, ethanol-water as diluent, shaking required to remove reddish hydrindantin color, new color factors for amino acids relative to leucine, etc. The modified reagent is prepared as follows:

4 N Sodium Acetate Buffer (pH 5.51 ± 0.03). Dissolve 2720 gm. of Na O Ac • 3 H_2O in 2 liters of H_2O on a steam bath. Cool to room temperature, add 500 ml. of glacial acetic acid and then H_2O to a volume of 5 liters. Final adjustment of pH, if necessary, is made with solid NaOH or glacial acetic acid. Store in the cold; preservative is not needed.

Reagent Solution. Dissolve 20 gm. of ninhydrin and 3 gm. of hydrindantin (Dougherty Chemicals) in 750 ml. of methyl cellosolve with gentle stirring. Add 250 ml. of pH 5.5 buffer. Transfer the resulting reddish solution immediately to a dark glass reservoir bottle and store under nitrogen. Prepare not more than a week's supply of reagent at one time.

Only the most alkaline buffers (pH 8.3, 9.2 and 11.0) used for the elution of basic amino acids in the 100 cm. column procedure (Figure 38) would still require adjustment of pH prior to color development with this reagent.

the base line both before and after the cystine peak. Choose the blank for cystine and valine after the valine peak. The blank reading usually changes somewhat with the emergence of the pH 4.25 buffer. Select the blank for methionine, isoleucine and leucine after the leucine peak.

In the 15 cm. column runs (Figure 39) the blank reading increases about 0.15 optical density unit when the pH 6.5 buffer emerges. Choose a blank for the ammonia peak just after the peak rather than before it. The blank usually falls again to its normal value before arginine emerges.

(h) *Calculation of Results*

Since the leucine calibration curve is plotted for volumes of 8.0 ml., volume corrections may be required in the calculation of results. Although the fraction collector is set to deliver samples of 1.0 ± 0.1 ml., it is advisable to spot check its performance by weighing every tenth tube in a run before and after a fraction has been collected. An approximate volume correction can then be made for any deviation from 1.0 ml. In addition, a correction may be made for the volume of acid or alkali used to adjust the solutions to pH 5. Because optical density readings are made at a minimal volume of 8.0 ml., these volume corrections are important only when maximum accuracy is sought.

Corrected optical densities are converted to "micromoles leucine" and the values for each peak are integrated or the sum of all the readings is used. Use of the appropriate color yield then converts these figures into micromoles of amino acid.

(i) *Preparation of Samples for Chromatograms*

Alanine-Cystine-Valine Mixture: The order and position of amino acid peaks emerging from the column, while quite reproducible under the conditions specified, are rather sensitive to changes of pH and ionic strength of the buffers. Of particular importance in this connection is the pH of the 3.42 ± 0.01 buffer. The position of the cystine peak varies markedly with small changes in pH of this buffer. At pH 3.47 cystine and alanine emerge almost together and at pH 3.37 cystine begins to overlap valine. It is necessary, therefore, to check the performance of the 3.42 buffer before use in analysis.

Prepare a solution in dilute HCl of 4 micromoles of alanine, 6 micromoles of valine, and 2 micromoles of cystine per ml. Mix 1 ml. of the solution with 1 ml. of pH 3.42 buffer and add 1 ml. of the mixture to the 100 cm. column. Run the chromatogram at 37.5° C. with the 3.42 buffer until valine is eluted (about 250 ml.). If the results show the peaks to be separated poorly, adjust the pH so that the cystine peak will be better positioned midway between the alanine and valine peaks, and repeat the experiment.

Amino Acid Mixture: It is important to run preliminary chromatograms with known mixtures of pure amino acids in order to establish a routine procedure whereby the reproducibility and accuracy inherent in the method can be realized. Unknown mixtures should then be analyzed in precisely the same way.

Prepare accurately a known mixture of amino acids and NH_4Cl simulating the composition of a protein hydrolyzate. Tryptophan may be omitted because the present method was developed for acid hydrolyzates. Cysteine may also be omitted because, if present, it is gradually oxidized and does not give a peak. The amounts of the 17 amino acids and NH_4Cl to be weighed out are determined by the following considerations. A total of about 1 g. of amino acids are dissolved in 1.5 ml. of 6 *N* HCl and made to 10 ml. with water. This solution may be stored in the cold. An aliquot of the solution is diluted 1 to 10 with pH 3.42 buffer for the 100 cm. column, or with pH 5.0 buffer for the 15 cm. column. A 0.5 ml. aliquot of the resulting solution will then contain about 5 mg. of total amino acids, a quantity suitable for analysis. The 5 mg. of total amino acids should include 0.1 to 0.5 mg. of each amino acid.

Recoveries of 100 ± 3% should be obtained for the majority of amino acids and ammonia. The recovery of glutamic acid is consistently about 97%, presumably because of slight conversion to pyrollidone carboxylic acid by the acidic solvents; a correction factor is used to compensate for this loss. Occasionally, slightly low recoveries (96%) of cystine and methionine are encountered.

(j) *Protein Hydrolyzates* (*cf.*, Chapter II)

With the development of methods for the precise analysis of mixtures of amino acids, increasing attention has been devoted to the conditions employed for the hydrolysis of purified proteins. It has been recognized that ordinary acid hydrolysis (6 *N* HCl, 16 to 24 hours, 100-120° C.) does not always liberate completely certain amino acids, such as valine and isoleucine, from resistant peptide linkages. It is also true that these conditions usually bring about complete destruction of tryptophan and variable decomposition of serine, threonine, cystine and, sometimes, other amino acids. For the determination of tryptophan, of course, some independent method of analysis, may be employed (*cf.*, Block and Bolling, 1951). In the case of the other amino acids, current practice is to use at least two

different periods of hydrolysis, for instance, 20 and 70 hours. Comparison of the analytical results provides information about both liberation and destruction of amino acids and permits closer estimates of the true amino acid composition of the protein. (Harfenist, 1953; Smith, E. L., 1954a, 1954b; Hirs, 1954b; Gordon, 1955.)

Hydrolyzates for analysis by the method under discussion should be prepared by heating with HCl, the excess acid should be removed by evaporation *in vacuo*, and the amino acid hydrochlorides should be mixed with pH 3.42 buffer before addition to the 100 cm. column or with pH 5.0 buffer for runs in the 15 cm. column. Usually, a 1 ml. aliquot of the hydrolyzate is mixed with 1 or 2 ml. of buffer, and a 1 or 2 ml. sample is added to the column. The following points may be considered before a more specific procedure for hydrolysis is adopted. It has been recommended that proteins be hydrolyzed in 6 *N* HCl redistilled in glass; that a large excess of acid (100 to 200 times the quantity of protein) be used to reduce humin formation; and that an even larger excess of acid be used in the presence of carbohydrate (Jacobsen, 1949; Stein, 1948; Schram, 1953; Dustin, 1953a,b). Hydrolysis is often carried out in evacuated, sealed tubes with adequate control of the temperature (Harfenist, 1953; Smith, E. L., 1954a; Hirs, 1954b), although in the case of crude proteins, hydrolysis under reflux is preferable (*cf.*, Chapter II). Results may be based on dry weight of protein hydrolyzed or on determinations of nitrogen in the hydrolyzate.

APPLICATIONS OF THE METHOD

1. Difficulties

The ion-exchange method (Moore, 1951) has been applied without major modification to the analysis of different proteins in many laboratories. The chief difficulty encountered in its use, insufficient resolving power in the cystine, glutamic acid-proline, and phenylalanine-tyrosine regions of the curve, may probably be attributed to the lack of uniformity of commercial samples of the resin. The continued collaborative efforts of the authors of the method and the manufacturers of the resin to provide more rigid specifications for particle size and degree of cross-linking may well help to overcome this difficulty (*cf.*, Moore, 1954a, footnote 1).

Even though some samples of resin used in the past years have not been fully satisfactory in resolving power, they have always yielded the same sequence of amino acids when eluted in the prescribed manner, and it has been possible to resort to various expedients to obtain usable results. For example, in analyses of insulins, Harfenist (1953) found that it was necessary to apply the Chinard acid ninhydrin method (1952) in the proline region for more accurate determination of this amino acid; that cystine could not be determined chromatographically; and that independent estimations of tyrosine were required to obtain values for phenylalanine from the combined tyrosine-phenylalanine peak. In analyses of prothrombin Laki (1954) used independent methods for tyrosine and phenylalanine, and determined cystine-cysteine chromatographically as cysteic acid after prior oxidation with performic acid. Similar supplementary procedures for the aromatic amino acids and cystine were required in analyses of muscle proteins (Kominz, 1954). Dual readings at 570 and 440 mμ were made for overlapping peaks of glutamic acid and proline in analyses of myoglobin and hemoglobin by Rossi-Fanelli (1955).

Poor resolution of the pairs glutamic acid-proline and tyrosine-phenylalanine occurs only occasionally and can be dealt with when it does. Spreading of the cystine peak is more serious. Not only is poor resolution of cystine sometimes encountered with synthetic mixtures of amino acids, but additional spreading of the peak occurs in protein hydrolyzates because of racemization (Hirs, 1954a, 1954b). Complicating the situation still further is the variable destruction of cystine during hydrolysis, especially in the presence of carbohydrate (*cf.*, Chapter II). For these reasons, determination of cystine by some other method is usually necessary. Often, performic acid* oxidation of the protein, prior to acid hydrolysis, is carried out with a twofold purpose: to permit estimation of cystine in the form of cysteic acid, which emerges unretarded at the hold-up volume (about 22 ml.) of the 100 cm. column (Laki, 1954; Smith, E. L., 1954a); and to permit more accurate definition of the alanine and valine peaks unobscured by cystine (Gordon, 1955). Conditions for the conversion of cystine to cysteic acid, and chromatographic procedures for the more certain identification and estimation of cysteic acid have been reported (Schram, 1954; Partridge, 1955).

The occasional low recoveries of methionine from known mixtures, presumably due to oxidation despite the routine use of thiodiglycol as an antioxidant in the eluting buffers, has been mentioned. Some oxidation of methionine may also occur during hydrolysis, especially

* Oxidation of cystine and cysteine to cysteic acid and methionine to methionine sulfone with peracetic acid in aqueous solution is preferable to performic acid (Block, unpublished).

in the presence of carbohydrate, with formation of methionine sulfoxide. When this happens, a sharp peak of the sulfoxide emerging just ahead of aspartic acid at pH 3.42 can be detected in chromatograms. The ninhydrin color of the sulfoxide is 102 relative to leucine (Schram, 1953). If the peak of sulfoxide is quantitatively significant, the methionine value can be corrected accordingly. This has been done in analyses of foods, such as cassava meal (Close, 1953a), barley, hay, and linseed presscake (Dustin, 1953b), cottonseed protein (Adriaens, 1954), human milk (Soupart, 1954), and also in analyses of virus protein (de Fremery, 1955).

Another difficulty, which has been troublesome at times, is variation in the density of ninhydrin color in the blank tubes. This is especially important when an amino acid is present in low concentration and it is essential for accurate work to choose a representative blank against which readings the peak are to be made. Variations in the blank can arise from absorption of ammonia from the air (Fraser, 1953) and from the presence of ammonia in reagents (Rossi-Fanelli, 1955). Variations in the blank may also be due to incomplete hydrolysis of a protein and the emergence of minor peaks of residual small peptides; longer periods of hydrolysis are required in this case (Smith, E. L., 1954a). Minor peaks in chromatograms of unknown mixtures are not necessarily artifacts and if it is desired to identify these or other peaks by paper chromatography the reader is referred to the papers by Stein (1953), E. L. Smith (1954a), and the book by Block, Durrum and Zweig (1955).

The ninhydrin color yields of amino acids relative to leucine have been checked by Moore (1951), E. L. Smith (1954a) and presumably by other users of the method. Occasionally, however, other values such as 90 for ammonia (Harfenist, 1953), 97 to 99 for glutamic acid and 97 to 98 for glycine (Simmonds, 1954), and 107 for lysine (Gordon, 1955) have been reported. The importance in this connection of running chromatograms of known mixtures, under the specific conditions employed by each investigator, is obvious.

Poor recovery (87%) of histidine from known mixtures was reported by Harfenist (1953). Reproducible determinations of arginine could not be obtained in analyses of virus protein (de Fremery, 1955). No explanations have been advanced for these observations.

In analyses of gelatin for ornithine by the 15 cm. column procedure Hamilton (1954b) found it necessary to change the pH, concentration, and sequence of some of the eluent buffers to give a more satisfactory resolution of histidine from hydroxylysine and, particularly, of ornithine from lysine. The same procedure was used for determination of hydroxylysine in collagen (Hamilton, 1955). The complete amino acid analysis of soluble proteins derived from elastin by Partridge (1955) included determination of hydroxyproline. This amino acid emerges well ahead of aspartic acid from the 100 cm. column at pH 3.04; because of the weak ninhydrin color yielded by hydroxyproline, color density was read against a separate calibration curve prepared with hydroxyproline itself. The use of the Troll (1953a) ninhydrin method for the estimation of hydroxyproline following chromatographic separation has also been proposed (Rogers, 1954; *cf.*, Chapter III).

2. Comments

The statements of Moore (1951), concerning the accuracy of the method when applied to known mixtures, have been substantiated

TABLE XI

AMINO ACIDS FOUND IN α-LACTALBUMIN HYDROLYZATES*

Amino Acid	Time of Hydrolysis					
	20 hrs.		70 hrs.		140 hrs.	
	H-5†	H-6	H-7	H-8	H-9	H-11
Aspartic acid	—	18.45	18.89	18.80	18.45	
Threonine	5.43	5.39	5.54	5.63	5.51	
Serine	4.70	4.70	4.73	4.88	4.79	
Glutamic acid	12.61	12.73	13.00	13.01	12.91	
Proline	1.52	1.64	1.53	1.48	1.43	
Glycine	3.07	3.12	3.25	3.23	3.32	3.24
Alanine	2.01	2.13	2.13	2.10	2.34	2.15
Cystine	4.37	5.60	3.58	4.33	3.05	6.66
Valine	3.20	3.25	4.60	4.52	4.89	4.62
Methionine	0.91	0.92	0.90	0.95	0.95	
Isoleucine	5.41	5.53	6.78	6.53	7.06	6.82
Leucine	11.09	11.44	11.67	11.54	11.93	11.43
Tyrosine	5.20	5.30	5.02	4.83	4.47	
Phenylalanine	4.36	4.27	4.47	4.62	4.64	

* The amino acid values are given as g. of amino acid yielded by 100 g. of anhydrous, ash-free protein.

† H-5 through H-11 designate individual hydrolyzates; H-11 was prepared after oxidation of the protein with performic acid.

Other comments: The figures for valine and isoleucine show that at least 70 hrs. of hydrolysis are needed for maximal yields of these amino acids. The yield of tyrosine decreases with time of hydrolysis. The figures for cystine illustrate the difficulties encountered in determination of this amino acid; the value of 6.66%, estimated as cysteic acid but expressed as cystine, is in fair agreement with independent determinations (6.4 ± 0.1%). The stability of serine and threonine in these experiments is noteworthy (Gordon, 1955).

in other laboratories. The reproducibility of results, when the procedure is applied to protein hydrolyzates, may be illustrated by some analyses of α-lactalbumin shown in Table XI (Gordon, 1955) and by similar tables for carboxypeptidase and papain (Smith, E. L., 1954a, 1954b).

Other proteins, not mentioned before, and some polypeptides which have been analyzed by ion-exchange column chromatography are presented in Part II.

Piez (1955) using a 100 x 0.9 cm. column of Dowex 50 at 50° C. and 6 ml. per hr. with 0.37 *M* citrate buffer at pH 3.1 (0.25 *N* sodium citrate) continuously varied by the addition of 0.25 *N* NaOH to give a ratio of flow rates in and out of the reservoir of 1:5.7 for 170 ml. and then a ratio of 1:10, found evidence of separation of isotopic (C^{14}) amino acids from their nonisotopic analogues.

3. Analysis of Amino Acid Solutions Not Obtained From Protein Hydrolyzates

For particular application of their procedures to mixtures of amino acids and related compounds more complex than protein hydrolyzates, Moore (1954a, 1954b; *cf.*, Partridge, 1952a; Sober, 1954; Thompson, 1953) has reported modifications of both the chromatographic separation and of the photometric ninhydrin method. A longer column (150 cm.), a 4% cross-linked resin (Dowex 50-X4), and an eluant of continuously changing pH and ionic strength are used to obtain a system with higher resolving power. A single chromatogram resolves 50 components almost completely. Modifications of the ninhydrin reagent include a fivefold increase in buffer strength to obviate the need for pH adjustment of the eluant, and replacement of stannous chloride by direct addition of hydrindantin. With the new method, analyses have been made of the free amino acids of blood plasma (Stein, 1954), tissue extracts (Tallan, 1954), and also of protein hydrolyzates (*cf.*, Part II). The chromatographic separations with Dowex 50-X4 are said to be more difficult to standardize than those with Dowex 50-X8.

4. An Automatic Procedure

A simplified chromatographic separation of amino acids together with an automatic recording apparatus for measuring ninhydrin color density have been described in a recent abstract (Spackman, D. H., Stein, W. H., and Moore, S., Federation Proceedings, *15*:358 (1956)). Two columns packed with fine mesh sulfonated polystyrene resin are required for complete quantitative amino acid analysis of a protein hydrolysate but only a single temperature (50° C.) and three eluent buffers are used, and the analysis is complete in 20 hours. The influent buffer is pumped through the column at a constant rate (up to 30 ml. per hour) by a stainless steel pump. The effluent from the column emerges in 1 mm. capillary tubing where it is mixed with a stream of ninhydrin reagent delivered at half the rate of the effluent by a second pump. The mixture passes through a 50 ft. spiral of 1 mm. -bore Kel-F tubing immersed in a boiling water bath where the ninhydrin color is developed. The optical density of the resulting solution is measured continuously as it flows through a cylindrical glass cell by three photometer units. Two of these measure densities at 570 mμ and the other at 440 mμ. The effective diameter of the glass cell is reduced from 2.2 to 0.8 mm. as it passes through one of the units measuring at 570 mμ in order to extend the range of the instrument when the optical density exceeds 1.3 with the larger cell. The optical density measured by each photometer is recorded by a Leeds and Northrop three-point recorder. The accuracy is 100 ± 3%.