The Use of Starch Chromatography and Ion Exchange Resin for Large Scale Separations of N¹⁵-Labeled Amino Acids

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In the past few years several methods have been worked out for the fractionation of amino acids and other organic compounds by means of the chromatographic technique. Since the first separation of N-acetyl-amino acids on silica gel by Martin and Synge¹, the field has been developed by various other workers.

The introduction of starch as the supporting media was made by Elsdén and Synge^{2,3}, and its usefulness has been demonstrated in the excellent procedures developed by Moore and Stein for the quantitative estimation of amino acids, both in synthetic mixtures and in protein hydrolysates⁴⁻⁸. With this technique it is possible to obtain almost all of the ordinary amino acids by two or three different separations. The original procedure was described and used only on a micro scale, for a few milligrams of protein, but it is also applicable to work on a larger scale, separating hydrolysates of 200—500 mg of protein.

The use of ion exchange resins for the separation of amino acids has also been extensively studied by several workers. Stein and Moore⁷ describe the use of Dowex 50 and point out that one of its advantages, as compared with starch, is its greater capacity, which allows the separation of gram amounts of amino acids.

In the first part of the present paper a technique is described for the separation, on Dowex 50 ion exchange resin, of large amounts of N¹⁵-labeled amino acids obtained from hydrolysates of biologically marked proteins. These labeled amino acids were used as tracers in later isotope experiments.

The second part presents a method of starch chromatography which has been used by us in these isotope experiments for the isolation of the common amino acids of tissue proteins, in amounts large enough to allow determination of the identity and purity of the different amino acids and their contents of N¹⁵.

EXPERIMENTAL AND RESULTS

The separation of N¹⁵-labeled amino acids on Dowex 50 ion exchange resin

The sources of N¹⁵-labeled amino acids were the proteins formed when yeast (*Torulopsis utilis*), in one experiment, and bacteria (*Escherichia coli*), in another, were allowed to grow on a medium in which the only source of nitrogen was an N¹⁵-labeled ammonium salt^{9,10}. The excess of N¹⁵ in the protein from *Torulopsis utilis* was about 17 % and in that of *Escherichia coli* 4.2 %. These biologically marked tissues were also used for the isolation of labeled components of nucleic acids. In the procedure of the experiment, the protein was taken as the residue which remained after extraction of the polynucleotides (Hammarsten¹¹) and treatment with hot trichloroacetic acid (Schneider¹²).

The protein was hydrolyzed by boiling in 6 N HCl on a hot sandbath for 24 hours. Excess hydrochloric acid was removed by repeated evaporations *in vacuo*; the residue was dissolved in distilled water and made up to a suitable volume.

In order to remove insoluble particles from the hydrolysate the sample was kept at -17° C. overnight and then thawed; after this treatment even the smallest particles were easily spun down in a centrifuge. The clear, brown supernatant was decanted; the hydrolysate was then ready for further use.

The first treatment of the hydrolysate was the removal of tyrosine by isoelectric precipitation. The hydrolysate was then divided into neutral, acidic and basic amino acids by means of electrodialysis according to the procedure of Sperber¹³. From the acid fraction glutamic acid was isolated as the hydrochloride and aspartic acid was precipitated as the copper salt. Both the basic fraction and the neutral fraction were further fractionated on Dowex 50 ion exchange resin.

The glass column used had a sintered glass plate at the bottom; it was 8 cm in diameter for the first 40 cm of length and 10 cm. in diameter above this. The ion exchanger which is available in very small particle size (250—500 mesh) was stirred up in 4 N HCl, poured into this glass column and allowed to settle. Washing with 4 N HCl was continued for a week or until about 10 l of acid had run through. The acid concentration was then changed to 1.5 N, which caused swelling of the resin and packed it in more tightly. The column was considered ready for use when the effluent had the same normality as the hydrochloric acid applied to the top. The length of the resin column was now 67 cm. The column was used at a temperature of 18° C.

Fig. 1. Separation of neutral amino acids on Dowex 50 ion exchange resin.

The amino acid mixture to be separated was dissolved in 1.5 *N* HCl and pressed into the column with slight air pressure. The walls were rinsed twice with a small amount of hydrochloric acid and the washings likewise pressed in. The column was then connected with a reservoir of 20 l of 1.5 *N* HCl. A slight pressure was applied to maintain a rate of effluent of about 175 ml per hour. Hourly fractions were collected with the aid of a time-controlled fraction collector, specially built at this laboratory for these large volumes. During the separation the hydrochloric acid concentration was first raised to 2.5 *N* and later on to 4 *N* in order to speed up the migration of the more slowly moving amino acids.

To localize the positions of the different amino acids the photometric ninhydrin method of Moore and Stein¹⁴ was applied to 0.05 ml of each fraction. The hydrochloric acid was neutralized with 0.2 ml of sodium hydroxide of a proper concentration, and the color was developed by the addition of 0.5 ml of the ninhydrin solution.

The separation of the neutral amino acids is shown in Fig. 1. In this experiment neutral amino acids equivalent to 1.4 g of nitrogen were applied to the column. It can be seen that the amino acids move out partly or completely separated from each other. The phenylalanine which is not plotted in the curve appeared in the effluent of 4 *N* HCl between 55 and 63 l.

In order to identify the different amino acids and to tell in which fractions two acids overlap each other an aliquot from every fraction was tested on a paper chromatogram and compared with known amino acid standards. A sample of 0.2—0.3 ml was evaporated to dryness on a small glass dish in an oven at 80° C. and then a few drops of distilled water was added and the process repeated three times to remove excess hydrochloric acid. The final residue was dissolved in a drop of water and applied to filter paper. These chromatograms were developed in different suitable solvent systems — *s*-collidine-water, *s*-collidine-water with diethylamine in the atmosphere, phenol-water and butanol-benzylalcohol-water (1 : 1 : 0.25). The chromatogram was always heavily overloaded in order to detect even very small traces of another acid.

From this picture of the distribution of the amino acids it was possible to judge which fractions contained a single amino acid, and to select pure fractions for almost all of these neutral amino acids. However, no fraction of methionine could be found which did not contain a trace of isoleucine. No significant amount of cystine could be detected; it is assumed to have been destroyed during the hydrolysis.

Fractions containing the same single amino acid were combined and evaporated to dryness *in vacuo*. The excess hydrochloric acid was removed by repeating the evaporation several times. The amino acids crystallized beautifully from the final residue without further purification. Since they had been isolated as hydrochlorides, the hydrochloric acid was removed with silver carbonate and the amino acids were recrystallized from water. The purity was again checked by means of paper chromatography; all the amino acids were found to be free from other ninhydrin-reacting substances except serine which contained a very slight and previously undetected trace of threonine.

The separation of the basic amino acids on Dowex 50 was similar to that of the neutral acids. The result is shown in Fig. 2. It can be seen that these amino acids separate very well from each other. However, since they emerge in such large volumes of 4 *N* HCl, there is some contamination by impurities from the acid and from the resin. Fractions containing the same amino acid were combined and evaporated as before; the residue was electrodialyzed and the free base isolated at the cathode. No further purification was performed.

With these separations it was possible to isolate the amino acids serine, threonine, glycine, alanine, valine, proline, isoleucine, leucine, phenylalanine, lysine, arginine, histidine and, by other means than Dowex, tyrosine, glutamic acid and aspartic acid. All of these acids were used as N¹⁵-labeled tracers in an investigation on interrelationships among different amino acids and between these and the formation of purines and pyrimidines. The isolation of amino

Fig. 2. Separation of basic amino acids on Dowex 50 ion exchange resin.

acids from tissue proteins in this study is described in the following section. Further details and results of these experiments will be presented in other publications.

The separation of N¹⁵-labeled amino acids by means of starch chromatography

For this and other investigations it was necessary to isolate the labeled amino acids from different tissue proteins and to determine their content of isotope. For this determination it is desirable to have amounts of each amino acid equivalent to 1—2 mg of amino acid nitrogen, which corresponds to 200—400 mg of protein. For the separation of amino acids on this scale a system of starch chromatography has been employed. This is the same as that used by Moore and Stein but on a scale about 80 times larger.

MATERIALS AND METHODS

Purification of the starch: For these experiments we have used potato starch, either from Morningstar Nicol, Inc.* or a commercial specimen of Swedish potato starch. Before use, the starch was extracted with methyl alcohol in a heated percolator at 55° C for 36 hours. The amount of alcohol necessary for this extraction is about 3—4 l per kg of starch. The material was then dried in a vacuum oven at 55° C for 24 hours.

Purification of the solvent: To commercial samples of n-butyl alcohol and n-propyl alcohol were added 100 ml of concentrated sulfuric acid per 10 l of solvent and the solvent was distilled *in vacuo* in a glass apparatus.

Preparation of the column: The first separation of the protein hydrolysate was performed on a column 8 cm in diameter filled with starch to a height of 30 cm. Subsequent re-separations were made with columns 4 cm in diameter with the same height of starch.

* Purchased from Amend Drug and Chemical Co., New York City.

Fig. 3. Separation on starch of liver protein hydrolysate. Column 8 cm in diameter, 30 cm high. Solvents, 1 : 2 : 1 butanol-propanol-0.1 N HCl, followed by 2 : 1 propanol-0.5 N HCl.

The extracted starch was stirred up in a mixture of butyl alcohol and water according to the directions of Stein and Moore⁵, then poured into the column and allowed to settle under an air pressure of 3–5 cm of mercury. When using starch with larger granules than those of the American sample, it has been found that more uniformly and tightly packed columns could be obtained if the starch was stirred up in butyl alcohol containing half as much water as that originally proposed.

The columns were then washed according to the directions of Moore and Stein⁶ to remove impurities which could be subsequently eluted by the developing solvents. When these solvents contained hydrochloric acid, the column was washed for 36 hours with a mixture of 1 : 1 propyl alcohol and 0.5 N HCl and then for 24 hours with the developing solvent itself before being used for chromatography. The one column for which no acid solvents were used (butanol-water being the eluting solvent) was treated instead with 8-hydroxy-quinoline.

Hydrolysis of the protein: 250–300 mg of protein were refluxed for 24 hours in a solution of equal parts of concentrated hydrochloric acid and 50 % formic acid. Excess acid was removed by repeated evaporations *in vacuo* and the final residue was made up to a volume of 10 ml. Insoluble particles were centrifuged down and the clear, brown supernatant was decanted. To 9 ml of this solution were added 1 ml of 1 N HCl, 20 ml of propyl alcohol and 10 ml of butyl alcohol. The resulting 40-ml mixture was applied to the starch column.

Separation and identification of the amino acids: The chromatograms were developed with different solvent systems, and hourly fractions of the eluate collected in the conventional way. The original separation employed first a solvent mixture of 1 : 2 : 1 butanol-propanol-0.1 N HCl, and later a mixture of 2 : 1 propanol-0.5 N HCl. Reseparations were made with 0.1 N HCl or with the butanol-water mixture as eluting solvent. All chromatograms were run under an air pressure of 7–10 cm of mercury.

The amino acids were localized by colorimetry in the same way as in the Dowex separation. The test was made on 0.2 ml of each fraction. With the 2 : 1 propanol-0.5 N HCl and the 0.1 N HCl solvents, 0.1 ml of sodium hydroxide of suitable concentration

Fig. 4. Reseparation on starch of valine, methionine and tyrosine. Column 4 cm in diameter, 30 cm high. Solvent, 0.1 *N* HCl.

was added to neutralize the hydrochloric acid, and the color was developed with ninhydrin in the usual way.

The positions of the individual amino acids were verified with standards run separately on identical starch columns. The volume of effluent corresponding to the top of each peak is reproducible, from one run to another, to within a few percent. Throughout the experiment the amino acids isolated were also identified and tested for contaminating amino acids and other ninhydrin-reacting substances by means of paper chromatography. The solvent mixtures used were the same as those mentioned above for the ion exchange procedure.

The result of the first separation is shown in Fig. 3. As mentioned above this separation was begun in a solvent mixture of 1 : 2 : 1 butanol—propanol—0.1 *N* HCl, and continued with a mixture of 2 : 1 propanol—0.5 *N* HCl.

It can be seen that several of the faster-moving amino acids overlap each other. The fraction containing valine, methionine, and tyrosine was reseparated on another column with an eluting medium of 0.1 *N* HCl. Under these conditions tyrosine is retarded and separated from valine and methionine. The separation is illustrated in Fig. 4. The mixed

Fig. 5. Reseparation on starch of leucine, isoleucine, methionine and valine. Column 4 cm in diameter, 30 cm high. Solvent, butanol saturated with water.

fraction of leucine and isoleucine from the first separation could be collected free from phenylalanine if some of the fractions in the valley were discarded. (The same was done to obtain pure threonine and aspartic acid.) Even when this was done, sufficient amounts of both fractions could be obtained. The leucine-isoleucine fraction was then added to the valine and methionine from the second chromatogram and the four acids separated on a third column in a butanol-water system as illustrated in Fig. 5. If traces of phenylalanine remained as a contamination of the leucine-isoleucine fraction, they could be detected on the paper chromatogram and removed in the reseparation with butanol-water, where the phenylalanine appears as a peak between leucine and isoleucine.

With these three different separations all the amino acids except alanine and glutamic acid are obtained in essentially pure form. These remaining two were separated by means of electrodialysis on a microscale, according to the directions of Theorell and Åkeson¹⁵.

When the glutamic acid and alanine fraction was tested on a paper chromatogram, the expected amino acids were identified besides four other definite ninhydrin-positive substances. Two of these were present in large amounts and moved very fast in both *s*-collidine and phenol. The other two gave faint spots and had slower travelling rates. None of these materials could be identified with any known amino acid or with any other of various ninhydrin-positive substances tested. It was considered possible that these compounds were formed when the glutamic acid and alanine fractions were evaporated to dryness in the alcohol-acid media before being tested with paper chromatography. The residue left after this evaporation was therefore hydrolyzed in hydrochloric acid, and the resulting solution again tested on a paper chromatogram. In this case only alanine and glutamic acid were found.

In order to isolate these substances, 500 mg of glutamic acid and 500 mg of alanine were refluxed in 500 ml of 1 : 2 : 1 butanol-propanol-0.1 *N* HCl on a hot sandbath for 3 hours. The solution was then evaporated to dryness *in vacuo*, dissolved in 40 ml of the above solvent mixture and applied to a starch column 8 cm in diameter and 30 cm high. Aliquots of the effluent were tested as usual with ninhydrin. The result is shown in Fig. 6.

The first material to emerge was slightly colored and is visible in the curve as a small peak labeled E_0 . In addition to this, four different peaks, partly separated from each other, were observed. The two fractions corresponding to the tops of peaks E_1 and E_2 were evaporated to dryness and tested with paper chromatography. They were identical with the two fast-moving components which gave strongly colored spots. Upon hydrolysis both gave rise to no ninhydrin-positive compound except glutamic acid. When glutamic acid was heated in butanol-acid the compound corresponding to peak E_1 was formed and with propanol compound E_2 . Acid hydrolysis of the materials from peaks E_3 and E_4 also produced only glutamic acid as amino acid constituent, but no further investigation was made to establish which alcohol takes part in their formation.

Both of the compounds labeled E_1 and E_2 have neutral properties during electrodialysis. The formation of these substances therefore introduces a hydrolysis of the dried glutamic acid-alanine fraction before these amino acids are separated by electrodialysis.

Aspartic acid has been tested in this same way but no substances like those formed from glutamic acid seem to be synthesized.

The only other contaminating ninhydrin-positive material found in these starch chromatograms in any significant amount was an unknown substance in exactly the same region as threonine. Its travelling rate on a paper chromatogram is about the same as that of γ -aminobutyric acid, but the two substances are not the same. All attempts to identify this material with known reference standards failed.

Fig. 6. Beginning of separation on starch of compounds formed by heating glutamic acid and alanine with 1 : 2 : 1 butanol-propanol-0.1 N HCl. Column 8 cm in diameter, 30 cm high. Solvent, 1 : 2 : 1 butanol-propanol-0.1 N HCl.

The substance was isolated free from threonine by a re-separation on Dowex 50. It is strongly retained on this resin and was eluted with the aid of 2.5 N HCl. The threonine, which moves fast (see Fig. 1), is well ahead of the unknown compound, which has a travelling rate about like that of arginine.

The substance was found in the neutral fraction when electro-dialyzed. It was only partly affected by hydrolysis for 24 hours in boiling 6 N HCl but was completely decomposed when heated in 6 N HCl in a sealed glass tube at 140° C for 48 hours. The hydrolysis products were separated on a starch column and identified as almost equimolar amounts of lysine and ammonia. The origin of this compound was established in an isotope experiment in which lysine labeled with N¹⁵ was injected into rats. From the liver proteins both the lysine and the unknown compound were collected. The latter was hydrolyzed as above and lysine and ammonia separated. Isotope determinations on these substances showed an excess of N¹⁵ in the lysine from protein which was exactly the same as that found in lysine isolated from the unknown compound. On the other hand, although the ammonia from protein contained a significant amount of N¹⁵, that from the unknown compound had none.

This indicated that both lysines were derived from the same source; the lysine in the unknown compound must have been that liberated in the hydrolysis of the protein, for both had the same excess of N¹⁵, and it is very unlikely that all lysine residues in the protein molecule have the same content of isotope. However, the ammonia in the unknown compound apparently comes from some external source, and it was concluded

that the compound itself was probably formed artificially during the treatment of the protein. When the polynucleotides are extracted from these tissues prior to the analysis of the protein, the materials are in one step treated with hot concentrated urea solution. A control experiment was performed in which a sample of lysine was heated with urea and the products separated on starch. Besides urea and lysine a considerable amount of the unknown compound was found. The urea necessary for the combination with lysine probably becomes attached to the protein in some way during this treatment with urea in the extraction of nucleotides.

Isotope determination and correction for foreign nitrogen: The total amount of nitrogen in each amino acid was determined on an aliquot of the sample by a semi-micro Kjeldahl procedure. The neutralized distillate obtained after titration in this determination was evaporated to a volume of about 1 ml, and the sample was then used for isotope determination. Nitrogen was liberated from the ammonium salt with the aid of alkaline hypobromite, according to the technique of Springson and Rittenberg¹⁶, and the excess of N^{15} estimated in either a 120° or 180° mass spectrometer.

Since each amino acid emerges in rather large volumes of solvent, a significant amount of non-amino nitrogen is derived from the solvent and the starch and is present in each sample. Some ammonia is also picked up by the acid solvents during the separation and the further treatment of the fractions. This foreign nitrogen will introduce a dilution of the isotope present in the amino acid nitrogen and a correction must be made for it. Our procedure for this has been as follows.

The actual amino acid nitrogen present in each case was calculated from the amount of α -amino nitrogen, which was determined on another aliquot of the amino acid sample by means of the Van Slyke procedure, as modified by Kendrick and Hanke¹⁷. Since about 25 % of the total nitrogen in ammonia is also set free in the Van Slyke estimation, ammonia must be removed prior to this analysis. This was done by distillation *in vacuo* in the presence of a slight excess of alkali. The ratio of the total amount of nitrogen found by the Kjeldahl procedure to the amino acid nitrogen calculated from the Van Slyke determination was taken as a measure of the dilution of the isotopic nitrogen and used as a correction factor. This factor varied from 1.05 to 1.25 for different amino acids and was estimated for each one separately.

In order to determine whether or not the foreign nitrogen contained any isotope, each of the amino acids was put through a Dowex 50 column with hydrochloric acid as the eluting solvent. Almost all of the foreign nitrogen was separated from the amino acid by this procedure and the factor determined by the same technique for the purified amino acids was much lower, ranging from 1.00 to 1.05. When a sample was tested for content of N^{15} both before and after this final purification on Dowex 50 and the isotope excess found in each case corrected by the respective factor, the two values for excess of N^{15} always agreed to within 1 %, indicating that the foreign nitrogen did not contain any appreciable amount of isotope.

The amino acid nitrogen from proline can not be determined by the Van Slyke reaction and isotope figures for this amino acid are therefore uncorrected for foreign nitrogen.

DISCUSSION

In general the procedures used in these separations of amino acids have been similar to those previously employed by Stein and Moore and already

discussed in their original publications. However, it has seemed desirable to modify their techniques in certain respects for this preparative work on a larger scale.

In the separation on Dowex 50 ion exchange resin, one of the chief modifications has been the separation of the protein hydrolysate into acid, neutral and basic amino acids before the fractionation on the ion exchange column. This was done for several reasons. If all the amino acids are put through the column together, aspartic acid, although well separated from glutamic acid, extensively overlaps the neutral amino acids serine and threonine. In addition phenylalanine, the neutral amino acid most strongly retained by the resin, appears as a flat peak in exactly the same place as the basic amino acid, arginine. Also, if electrodialysis is introduced, the original amide nitrogen, which has been converted to ammonia, is aerated away during this procedure, so that the amino acid valine emerges without any contamination of ammonia, which otherwise would appear close in front of it in the eluate. Thus, although the electrodialysis introduces an additional procedure, it enables one to obtain much cleaner separations of several of the amino acids and seemed to be a necessary addition for a fractionation of the amounts³ of protein hydrolysates we have worked with here.

The amounts of amino acids separated on Dowex in our experiments are approximately 100 to 300 times larger than those of the Stein and Moore separation, and our columns, therefore, have probably been heavily overloaded. This may partly explain our less successful separation of methionine, isoleucine and leucine. However, another explanation may be the fact that this separation has been carried out at a somewhat lower temperature (18° C.) than the other, which was run at 25° C. The temperature effect has been discussed in detail by Moore and Stein⁷ and by Partridge and Brimley¹⁸. However, except for this fractionation, the separation appears to be quite satisfactory, even on this large scale.

Our starch separation has also been carried out on a larger scale than that of Stein and Moore, but this has been accomplished by the use of larger columns, so that the proportions of starch used are approximately the same. The resolving power of these large starch columns is as good as that of the smaller columns described in the original method. The amounts of amino acids should not exceed 500 mg for an 8-cm column if clean-cut separation is to be obtained for the amino acids which emerge close together. The amounts which can easily be accommodated in the re-separations are comparatively larger, and the amounts of leucine, isoleucine, valine, methionine and tyrosine obtained from the separation on an 8-cm column can be cleanly re-separated on a column of half this diameter.

Before the starch columns can be used for the separation of amino acids they must be freed of organic impurities and metal ions. This is performed by allowing alcohol-acid solvents to pass through the column for 36 hours. This washing is enough to extract from the starch the metal ions and the soluble nitrogenous impurities, but there still remain appreciable amounts of fatty material which are later eluted into the amino acid fractions and can be seen as a fatty residue after the sample has been evaporated down to dryness. Washing the column for a longer time with alcohol-acid lowers the resolving power of the column, and it has therefore been found preferable to remove this fatty material by extracting the starch prior to its use for columns with a better fat-extracting solvent, namely methyl alcohol.

Although these washings remove most of the impurities from the starch, there are also impurities in the solvents themselves, so that amino acid fractions isolated from the starch separation contain appreciable amounts of extraneous nitrogen, as shown by the differences between total nitrogen and amino nitrogen determinations. Although it has been demonstrated that this foreign nitrogen does not contain any significant amount of N^{15} , it is undesirable from several standpoints to have to deal with such high correction factors. In more recent work, therefore, the reseparation of each amino acid on Dowex 50 has been introduced as a routine procedure. The removal of ammonia prior to the Van Slyke estimation can then be eliminated, since the Dowex columns can be made of proper length to give a satisfactory separation of ammonia from each amino acid.

The solvent system suggested by Stein and Moore⁵ for the separation of the first six amino acids — leucine, isoleucine, phenylalanine, methionine, tyrosine and valine — was found to be somewhat unsatisfactory for a separation on this larger scale, in that the separations were not reproducible from one run to another. In addition it was very difficult to free the benzyl alcohol which was available from nitrogenous impurities, and it was extremely time-consuming to redistill the necessary large volumes of this alcohol because of its high boiling point. We have, therefore, used instead a solvent system of acid-propanol-butanol, even though this introduces the necessity of reseparating five of these amino acids.

The reseparation of valine, methionine and tyrosine has been simplified in later work in this laboratory by the use of Dowex 50 instead of starch. Hydrochloric acid is used as eluting solvent and all three amino acids are isolated free from each other in a single run. In a similar manner it is easier to reparate alanine and glutamic acid on Dowex 50 rather than by use of electrodialysis. This modification is also currently in use in our laboratory. Still another application of ion exchange separation has been in the isolation of

threonine from aspartic acid. If the overlapping of these two amino acids is extensive enough to cause large losses of material, it is better to reseparate them on the anion exchange resin, Dowex A 1. We have found that such combinations of starch chromatography with ion exchange separations are frequently the simplest and most effective way to separate amino acids on this scale.

Throughout these experiments the identification of the different amino acids obtained from both the ion exchange and the starch separations has been made by means of paper chromatography. Nearly all of the solvent systems used for this technique are well known from previous work. The addition of diethylamine to the atmosphere of the *s*-collidine-water system improved the separation between phenylalanine and the two leucines isolated from starch. The mixture of butanol-benzylalcohol-water was used for the identification of the mixed fraction of leucine, isoleucine and methionine from Dowex 50.

Calculations of the quantities of amino acids separated from the proteins have been made both from the light absorption of the color developed with ninhydrin (for an aliquot of each fraction) and from the amino nitrogen determined by Van Slyke on the isolated amino acids. The calculations from the ninhydrin figures were made according to the method of Moore and Stein¹⁴. A calculation of recovery of nitrogen can be made from these values, although it is only approximate because of error due to overlapping of amino acids in some fractions. By both methods such a calculation indicates the total nitrogen recovered to be 85—95 % of that present in the original hydrolysate. When a synthetic mixture of amino acids has been analyzed the nitrogen has been recovered quantitatively. It appears therefore that there may be some nitrogen present in the trichloroacetic-acid-precipitated protein which is not amino acid nitrogen, although the error in the calculations makes exact figures questionable.

The form in which this nitrogen exists is unknown, for this figure for recovery of nitrogen is based not just on amino acids isolated but on all nitrogen-containing materials that we have been able to obtain from the hydrolysate. Thus during the hydrolysis of the protein a dark brown, acid-insoluble, solid material is formed and centrifuged off from the hydrolysate. This material is named acid-insoluble humin. The brown supernatant, when applied to the column, gives rise not only to individual amino acids, but also to a brownish-colored constituent which moves very fast, almost at the solvent front. This is termed acid-soluble humin. These two substances, formed during the acid hydrolysis, contain decomposition products of certain of the amino acids and of carbohydrates. The nitrogen contents of both of these were always

determined and taken into consideration in the calculation of the recovery figure. Both were also tested for content of N¹⁵, but no attempt was made to fractionate these humins or to identify their constituents.

Although cystine should be among the neutral amino acids isolated by these techniques, we have never found appreciable quantities of this amino acid in eluates from either type of column. The amounts of methionine found have also been low, actually too low to allow an accurate determination of amino nitrogen or of content of N¹⁵. This is thought to be due to partial destruction of cystine during hydrolysis and to a very low content of both of these sulfur amino acids in the liver protein analyzed. Since the use of acid as hydrolyzing agent also destroys all of the tryptophane, it is not surprising that no trace of this amino acid has ever been found. These disadvantages of acid hydrolysis of proteins are well known, but, on the other hand, no method is completely satisfactory, and acid hydrolysis has an advantage in simplicity. It has been considered quite adequate for our purposes in the present experiments.

From the present results it seems highly probable that the four ninhydrin-positive substances found in the glutamic acid-alanine region on the starch chromatograms are different esters of butyl and propyl alcohols with glutamic acid. The two present in greatest quantities, corresponding to peaks E₁ and E₂ in Fig. 6, are most likely the monoesters, since they have neutral properties in electrodialysis. Other kinds of esters of glutamic acid with these alcohols can probably explain the formation of E₃ and E₄. It is quite possible that these substances are the same as those observed by Borsook¹⁹.

In the Dowex separation of amino acids from the protein of *Escherichia coli* two small peaks were observed ahead of the first amino acid, aspartic acid. The constituents of these fractions were both crystalline substances which gave a slight reaction with ninhydrin; their identity has not yet been established. These unknown substances have also been observed by Ehrensvärd²⁰ in a similar analysis of hydrolysates of *Escherichia coli* protein.

SUMMARY

Methods previously described by Stein and Moore for the separation of amino acids have been satisfactorily applied in the present experiments to preparative work on a larger scale. All of the common amino acids have been isolated from acid hydrolysates of 25—50 grams of protein by means of the cation exchange resin Dowex 50 with hydrochloric acid as eluting solvent. Starch chromatography has been employed to separate the same amino acids from hydrolysates of 250—300 milligrams of protein. Both techniques have

been used to isolate samples of N¹⁵-labeled amino acids, and have been found quite satisfactory for the isolation of materials with the degree of purity required for isotope work. The identity of the amino acids isolated has been proved by means of paper chromatography. The existence of several other unknown substances has been revealed by these separations, and the identity of these substances is discussed.

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