

frared spectra of the two samples in chloroform were identical.

**Methanolysis of Cevadine to Protocevine. A. At Room Temperature.**—Cevadine (500 mg.) was dissolved in a mixture of methanol (15 ml.) and water (5 ml.) and the solution was allowed to stand for five days at room temperature. The solvents were then concentrated until the solution became turbid. Seeding with cevadine led to the separation of crystalline starting material (440 mg.), m.p. 210–212°. The filtrate was evaporated to dryness *in vacuo* and benzene was added and boiled off to remove the last traces of water. The amorphous residue was boiled with ether (30 ml.) and filtered from insoluble solid. Concentration of the filtrate led to the separation of protocevine (30 mg.), m.p. 220–225° dec. after sintering from 175°.

**B. At Reflux Temperature.**—Cevadine (2 g.) was dissolved in a mixture of methanol (10 ml.) and water (5 ml.) and the solution was heated under reflux for five days. Concentration of the solvents and seeding with cevadine led to the recovery of 390 mg. of unchanged starting material. The filtrate was evaporated to dryness *in vacuo* and dried by adding and boiling off benzene. The amorphous residue crystallized from chloroform-ether, yielding cevagenine (75 mg.), m.p. 245–248° dec. after sintering from 170°. Evaporation of the filtrate to dryness left a residue which crystallized from ether yielding protocevine (260 mg.), m.p. 220–225° dec. after sintering from 170°; infrared spectrum in chloroform identical with that of the analytical sample above.

**Alkaline Hydrolysis of Cevadine to Protocevine.**—To a solution of cevadine (5.92 g., 0.01 mole) in methanol (150 ml.) and water (60 ml.) was added 4% sodium hydroxide solution (10.1 ml., 0.011 mole) and the solution was heated under reflux for 10 minutes. The slightly yellow solution was cooled and made just acid with 1:1 hydrochloric acid and the methanol was evaporated *in vacuo*. To the residue, cooled to 0–5°, was added 10% sodium hydroxide solution to neutrality and then aqueous ammonia to pH 9. The alkaline suspension was extracted with chloroform (four 50-ml. portions) and the chloroform solution was washed and concentrated to 30 ml. Addition of ether (20 ml.) led to separation of cevagenine (1.0 g., m.p. 245–248° dec. after sintering from 170°). Evaporation of the filtrate to dryness left a residue which crystallized from ether (1.68 g.). Recrystallization from ether gave protocevine (1.39 g.), m.p. 220–225° after sintering from 175°; infrared

spectrum in chloroform identical with that of the analytical sample above.

**Alkaline Hydrolysis of Veratridine to Protocevine.**—Veratridine (6.73 g., 0.01 mole) was treated with dilute methanolic sodium hydroxide and the reaction mixture worked up as described above for the alkaline hydrolysis of cevadine. From the chloroform-ether solution, cevagenine (0.81 g.) was obtained. From the residue after removal of cevagenine, 1.9 g. of crude crystalline product was obtained from ether. Recrystallization from the same solvent yielded protocevine (1.53 g.), m.p. 220–225° dec. after sintering from 179°; infrared spectrum identical in chloroform with that of the analytical sample above.

**Anhydrocevadine Triacetate.**—Cevadine was acetylated with acetic anhydride and perchloric acid according to the procedure described by Stoll and Seebeck.<sup>8</sup> Recrystallization of the product from ether gave rectangular plates, m.p. 278–280° dec.,  $[\alpha]_D^{+90}$  (c 2.15, chf.).

*Anal.* Calcd. for  $C_{38}H_{53}O_{11}N$ : C, 65.22; H, 7.63. Found: C, 65.64; H, 7.80.

In a volatile acid determination<sup>11</sup> 35.96 mg. of substance yielded an amount of acid equivalent to 24.45 ml. of 0.008103 *N* sodium thiosulfate; calcd. for anhydroprotocevine triacetate monoangelate, 25.37 ml.

**Cevadine Diacetate.**—Cevadine was acetylated with acetic anhydride and pyridine according to the procedure of Stoll and Seebeck.<sup>8</sup> Recrystallization of the crude product from ether-petroleum ether yielded colorless needles, m.p. 258–260° dec.,  $[\alpha]^{25D} -13^\circ$  (c 2.17, alc.).

**Cevagenine Triacetate.**—Cevagenine (3.0 g.) was acetylated with acetic anhydride and pyridine at room temperature according to the procedure of Stoll and Seebeck.<sup>8</sup> Crystallization of the crude product from ether afforded anhydrocevagenine triacetate (prisms, 1.08 g., m.p. 285–286° dec.). Concentration of the filtrate led to the separation of cevagenine triacetate in the form of needles (0.93 g.). Repeated crystallization from acetone-ether gave needles (0.45 g.) which melted at 242–243.5° dec.,  $[\alpha]^{25D} -62^\circ$  (c 1.33, alc.).

**Anhydrocevagenine Triacetate.**—Cevagenine was treated with acetic anhydride and pyridine at steam-bath temperature according to the directions of Stoll and Seebeck.<sup>8</sup> The product melted at 285–287° dec.,  $[\alpha]^{25D} -47^\circ$  (c 1.17, alc.).

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## Isolation of Amino Acids by Distillation of the Acetylated Amino Acid Ethyl Esters

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Mixtures of acetylated amino acid ethyl esters were prepared from protein hydrolyzates, and the mixed esters were fractionated in efficient distilling columns. The esters are sufficiently stable to withstand efficient fractionation procedures. Fractions rich in alanine, valine, leucine, isoleucine, proline, aspartic acid, glutamic acid, methionine and phenylalanine were obtained under moderate fractionation. By more precise fractionation, alanine, valine, leucine and isoleucine were obtained as the pure amino acids. These pure amino acids retained an appreciable percentage of their original optical activity.

### Introduction

The slow development of a commercial amino acid industry as compared with the rapid development of the fatty acid industry is probably due in large part to the difficulty and expense involved in isolating or synthesizing the amino acids in quantity. General technological advances in recent years have indicated that further knowledge of the separation of the volatile derivatives of the amino acids may lead to an economical means of producing the amino acids from proteinaceous agricultural products.

The work of Fischer<sup>2</sup> on the distillation of the amino acid ethyl esters aroused considerable interest for a time because it appeared to offer an excellent means of separating the amino acids from a protein hydrolyzate. A wide variety of proteins were subjected to analysis by this method, but the results were far from satisfactory. Each of the fractions obtained contained a mixture of all the amino acids present, and a considerable residue—apparently amino acid anhydrides—remained in the still pot.

Morgan<sup>3</sup> prepared the butyl esters of some of the amino acids and claimed that they were consider-

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

(2) E. Fischer, *Ber.*, **34**, 433 (1901).

(3) W. T. J. Morgau, *J. Chem. Soc.*, 79 (1926).

ably more stable than the ethyl esters. Layton<sup>4</sup> undertook to use the distillation of these butyl esters as a means of fractionating protein hydrolyzates in efficient distilling columns. He showed that the boiling points were sufficiently different to enable fractionation on suitable columns, and he obtained stepwise distillation curves. He was unable to obtain more than two amino acids as pure fractions, however, and about 30 to 40% of the starting weight was left as a non-distillable residue. Apparently these butyl esters are not stable enough to withstand lengthy distillation procedures.

The unstability of the ethyl esters led Cherbuliez and Plattner<sup>5</sup> to investigate the acylated amino acid ethyl esters. They prepared most of the acetylated esters of the naturally occurring amino acids and obtained their approximate boiling points at pressures of a few mm. They did not describe any attempts to make or fractionate the mixed acetyl esters obtained from a protein hydrolyzate.

The acetylated amino acid ethyl esters did not appear to have the condensing or polymerizing properties of the amino acid esters, and an attempt to separate them by fractional distillation appeared promising.

Accurate vapor pressure-temperature curves between 2 and 90 mm. pressure were prepared<sup>6</sup> for most of the acetylated amino acid ethyl esters. The spacing of these curves showed that in most instances distillation should be an effective means of separating the esters.

Experiments were then started to prepare the mixed acetylated ethyl esters of the amino acids in protein hydrolyzate and to fractionate this mixture by distillation. When the mixture had been adequately prepared for the distillation, the process had few complications. Suitable preparation of the mixed acetylated ethyl esters is a troublesome problem, which has not been adequately studied. Only one of the many possible methods of preparation is described here, and the yield by this method is low. Sufficient material, however, was produced to test the applicability of fractional distillation to the separation of the acetylated amino acid ethyl esters.

### Experimental

**Preparation of Materials.**—Mixtures of amino acids were prepared by hydrolyzing proteins with sulfuric acid. The acid was neutralized with an excess of lime, and the residual lime in the solution was removed with oxalic acid. The amino acid solution was concentrated to a small volume and adjusted to pH 9.0 with sodium hydroxide. After cooling to 0°, this solution was acetylated with acetic anhydride; the pH was kept constant at 9.0. After the reaction was completed, the mixture was acidified to congo red with sulfuric acid and then evaporated to incipient crystallization. It was allowed to stand at 5° overnight. The crystallized sodium sulfate was then filtered off, and the liquid was evaporated to a small volume. The small amount of dissolved sodium sulfate was precipitated by the addition of two volumes of absolute alcohol and removed by filtration. After evaporation of the alcohol *in vacuo*,

the residue was again dissolved in absolute alcohol and concentrated *in vacuo* to remove most of the water present.

The residue of acetylated amino acids was then dissolved in absolute alcohol, acidified with several ml. of concentrated sulfuric acid, diluted with an equal volume of benzene, and refluxed. The vapors produced were passed through a seven-ball Snyder column and collected in a fractionating receiver. The lower aqueous layer was withdrawn, and the upper layer was returned to the column. When two layers ceased to form in the condensate, esterification was considered complete, and the benzene and alcohol were distilled off *in vacuo*. These mixed acetylated esters are easily converted to the azlactones at elevated temperatures in the presence of strong acids. The concentration was, therefore, carried out below 50°, and a small amount of alcohol was allowed to remain in the residue to prevent formation of azlactones.

The concentrated alcohol solution was dissolved in ether. The ether solution was neutralized with anhydrous ammonia, and the precipitated solids were filtered off. The ether was evaporated, and the oily residue was distilled rapidly in a Claisen-type vacuum still. The residual volatile solvents were removed in this distillation, and the acetylated amino acid esters were obtained as a viscous light-colored oil.

In the preparation described, almost quantitative acetylation is obtained, as shown by the drop in Van Slyke nitrogen. The acetylated amino acid mixture is difficult to separate from the accompanying salts, and some hydrolysis of the acetyl derivatives occurs during the concentration. Other extraction solvents have been tried, but none has appeared promising. Additional splitting of the acetyl groups also occurs during the esterification, and the liberated acetic acid distills off as ethyl acetate. This appears to disrupt the formation of the benzene-alcohol-water azeotrope and produces a false end-point to the esterification process. Acetic acid, which may also be carried over into the esterification mixture, would also have this effect. The esterification product, therefore, probably contains acetylated and unacetylated amino acid esters and acetylated amino acids.

The ether extraction before neutralization of the catalyzing acid eliminates most of the undesirable materials, but this procedure encourages the formation of azlactones. The alcohol left in the concentrated esters to prevent this formation of azlactone complicates the Claisen distillation to isolate the purified mixed acetylated amino acid ethyl esters.

In the procedure described, glutamic acid would appear as the acetylated diester. This is not the derivative most suitable for the distillation because this ester is pyrolyzed to pyrrolidone carboxylic acid ethyl ester at the temperature of the distillation. The ethyl acetate produced in this pyrolysis upsets the pressure regulation during the Claisen distillation. This pyrolysis may also cause decomposition of some of the other bifunctional amino acids. In most cases when this pyrolysis occurred, no undistillable residue was obtained; however, a number of preparations produced considerable non-distillable material.

**Distillation.**—Separations obtained in any distillation depend to a large extent upon the nature of the fractionating column and the method under which it is operated. The fractionations reported here can readily be divided into two sections, based on the fractionation technique. In the first section, the mixture of acetylated amino acid esters was fractionated in a laboratory fractionation assembly designed by Todd.<sup>7,8</sup> The column was operated at a low reflux ratio (3-1) and a high rate of take-off (1 ml./min.) to separate the acetylated ester mixture obtained from a protein hydrolyzate. The 25 mm. column was packed with  $\frac{3}{16}$  inch diameter Pyrex helices to produce the data of Fig. 1 and with  $\frac{3}{32}$  inch helices to produce the data of Fig. 2.

In the second section, selected fractions obtained from these distillations in the Todd column were combined and distilled in a 13 mm.  $\times$  36 inch Podbielniak<sup>9</sup> column at a high reflux ratio (60-1) and slow rate of take-off (5 ml./hr.) to produce the data for Figs. 3 and 4.

**Analysis.**—The fractions obtained from each fractionating column were analyzed for their amino acid composition

(4) L. L. Layton, Ph.D. Thesis, Penna. State College, Aug., 1942 (Pub. No. 518, Univ. Microfilms, Ann Arbor, Mich.).

(5) E. Cherbuliez and Pl. Plattner, *Helv. Chim. Acta*, **12**, 317 (1929).

(6) E. F. Mellon, S. J. Viola and S. R. Hoover, *J. Phys. Chem.*, **57**, 607 (1953).

(7) F. Todd, *Ind. Eng. Chem., Anal. Ed.*, **17**, 175 (1945).

(8) Mention of a product does not imply that it is endorsed or recommended by the U. S. Department of Agriculture over similar products not mentioned.

(9) W. J. Podbielniak, *Ind. Eng. Chem., Anal. Ed.*, **3**, 177 (1931).

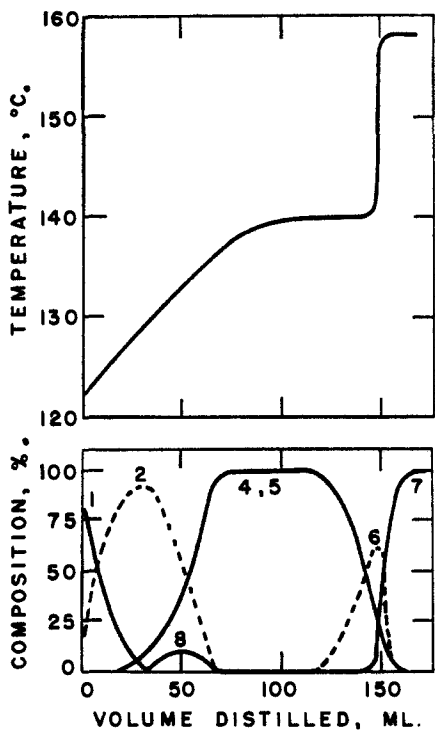


Fig. 1.—Distillation and composition curves for the fractionation in a Todd still at 4.5 mm. pressure of a mixture of the acetylated ethyl esters of: 1, alanine; 2, valine; 4, isoleucine; 5, leucine; 6, proline; 7, aspartic acid; 8, glutamic acid.

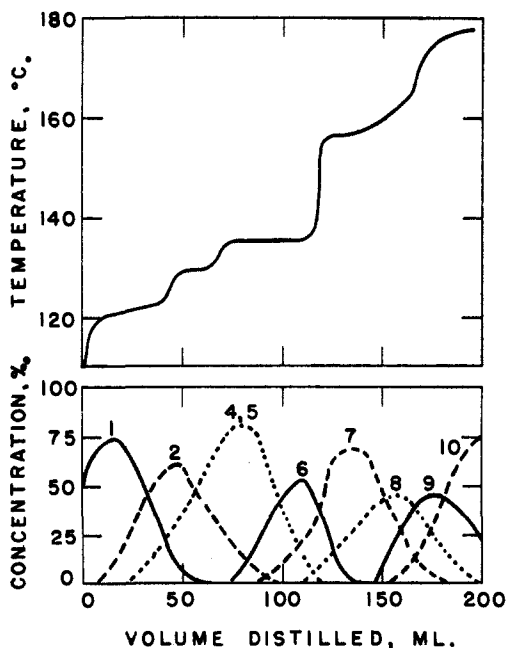


Fig. 2.—Distillation and composition curves for the fractionation in the Todd still at 3.8 mm. pressure of a mixture of the acetylated ethyl esters of: 1, alanine; 2, valine; 4, isoleucine; 5, leucine; 6, proline; 7, aspartic acid; 8, glutamic acid; 9, methionine; 10, phenylalanine.

by paper chromatography. The samples (0.1 g.) were hydrolyzed in 10 ml. of 20% HCl for 24 hours. The hydrolyzate was concentrated to dryness, dissolved in 77% alcohol, and neutralized with several drops of 4 *N* NaOH.

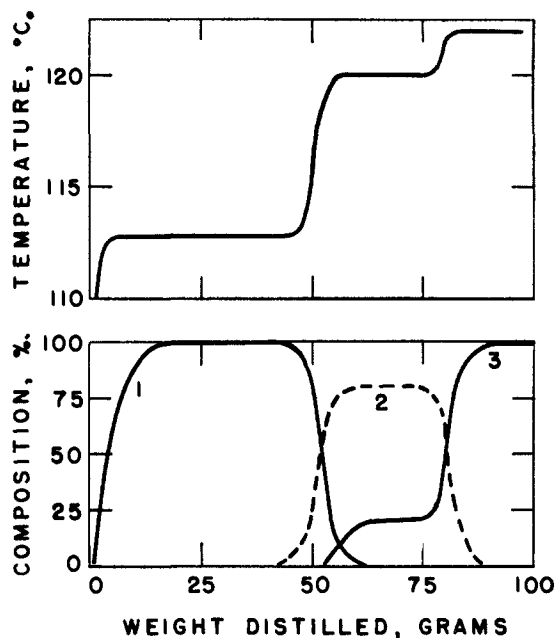


Fig. 3.—Distillation and composition curves for the fractionation in a Podbielniak still at 3.8 mm. pressure of a mixture of the acetylated ethyl esters of: 1, alanine; 2, valine; 3, glycine.

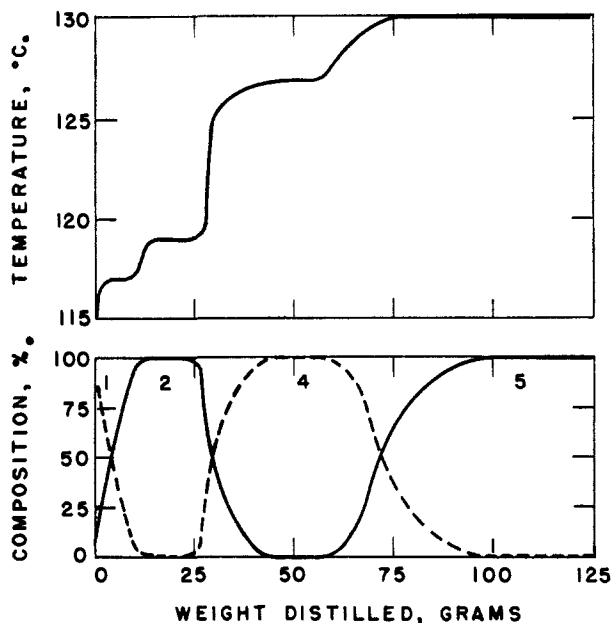


Fig. 4.—Distillation and composition curves for the fractionation in a Podbielniak still at 3.8 mm. pressure of a mixture of the acetylated ethyl esters of: 1, alanine; 2, valine; 4, isoleucine; 5, leucine.

The mixture was filtered, and diluted to 10 ml. with 77% alcohol. A 0.002-ml. aliquot of this solution was placed 1 inch from the edge of an 11 × 14 inch sheet of Whatman no.1 filter paper. About six unknown spots and six suitable control spots were run on each sheet. The sheets were made into a cylinder and developed in an ascending direction in 6 × 18 inch Pyrex cylinders.

The atmosphere was saturated with the water phase of the solvent by a central wick of filter paper. Two 1-dimension chromatograms, one developed with phenol saturated with water and the other developed with collidine saturated with water were sufficient to separate the mixture except when the leucines were present. Leucine and isoleucine

were separated from each other on descending chromatograms by developing with *t*-amyl alcohol saturated with water. Three days' development, with the solvent dripping from the end of the paper, were required to make the spots travel about 8 inches, but a separation of about 1 inch between the two spots was obtained.

The sheets were sprayed with 0.1% ninhydrin in butanol and dried at 70°; they were then steamed with live steam, resprayed with the ninhydrin solution, and again dried at 70°. The area of the spots was determined with a planimeter, and the concentration of the amino acid in the solution was determined from a standard plot of the area against the log of the concentration. Leucine solutions were used to make this standard plot. It can be used to determine all the amino acids, except proline, to within 10 to 15%.

The composition values for each fraction were then plotted, to give continuous composition curves. The trace spots of some amino acids were neglected because they indicated that only insignificant amounts of these materials were present.

The fractions that gave chromatographic evidence of only one component were analyzed for total nitrogen by the Kjeldahl method. A fraction of each acetylated amino acid ester that gave a satisfactory nitrogen analysis was hydrolyzed to regenerate the amino acid, which was then recrystallized and dried. The optical rotation of these crystals was determined in 2 or 6 *N* hydrochloric acid.

### Results and Discussion

Although difficulties were encountered in the preparation of the mixed acetylated amino acid ethyl esters, sufficient material was obtained to show that fractional distillation will separate many mixtures of them into the pure components.

Figure 1 shows the distillation curve for a mixture of acetylated ethyl esters obtained from the hydrolyzates of 2 kg. of air dried casein. There is no sharp fractionation at the low temperature end of the boiling point curve. The composition diagram, however, indicates that some fractionation is occurring, although considerable overlapping of the composition curves exists. For some 40-ml. volume obtained in the center region of the curve, the composition was almost completely that of the two leucine derivatives. In the higher temperature region, there was a sharp fractionation between the proline and aspartic acid derivatives. The small amount of glutamic acid derivative occurring between the valine and leucine appeared to be due to some derivative of glutamic acid other than the acetylated diester or pyrrolidone carboxylic acid ethyl ester, for its boiling point seemed to be about 15° below the lowest of these expected derivatives.<sup>6</sup> A similar phenomenon occurred in the distillation of most mixtures containing glutamic acid, although most of the glutamic acid appeared to distil as the pyrrolidone carboxylic acid ethyl ester. The low yield of distillate (165 ml.) from 2 kg. of casein was due mainly to losses preceding the fractional distillation step. Only 180 ml. was introduced to the still-pot and about 7 ml. remained in the pot after the distillation.

Figure 2 shows the distillation curve for a mixture of acetylated ethyl esters distilled in the Todd column packed with smaller helices and with the take-off set at a slightly slower rate. Use of smaller helices and slower rate of distillation improved the fractionation, as indicated by the greater number of steps in the distillation curve. There is still considerable overlapping of the composition curves for the various components. In this distillation, the glutamic acid derivative appeared mainly as

pyrrolidone carboxylic acid ethyl ester, although a trace of the material came over between the valine and leucine fractions. This curve shows, however, the fractionation into pure components is possible if a more efficient column and larger volumes of each component are employed.

To test the separation of these acetylated amino acid ethyl esters under more promising conditions, a mixture of the alanine and valine derivatives obtained from previous fractionations was combined with a sample of synthetic acetylglycine ethyl ester, and this mixture was distilled in a Podbielniak column. Figure 3 shows that an excellent fractionation was obtained. The first cuts contained some unidentified material of high volatility that crystallized in the receiver. After this was distilled off, a number of cuts of the alanine derivative were obtained, which on hydrolysis gave chromatographically pure alanine. After the transition region, a mixture of essentially constant composition (80% valine derivative and 20% glycine derivative) was obtained. This indicated an azeotropic mixture, but this phenomenon has not been investigated fully. After all the valine derivative had been removed a number of cuts containing the pure glycine derivative were obtained. There is a difference of 2 degrees in distillation temperature between these two fractions, although there is only a half-degree difference in boiling point between the pure materials.<sup>6</sup>

Another batch of selected fractions, containing only the alanine, valine, isoleucine, leucine and proline derivatives, was fractionated in the Podbielniak column under the same conditions as in the previously described fractionation. The distillation curve shows four transitions, and the composition curves show that these transitions were between pure fractions of the four components. There was insufficient alanine derivative to make a pure fraction of this material, but its separation from the valine derivative was shown in the previous example. The proline derivative remained in the still-pot and column. Chromatographically pure fractions of the valine, isoleucine and leucine derivatives were obtained. This separation of a mixture of the leucine and isoleucine derivatives into the two components, each free of the other, is rarely accomplished by other means. Nitrogen determinations made on these pure fractions to check the paper chromatographic evidence of purity showed the following results: valine derivative, theoretical value, 7.48%; found 7.61%; isoleucine derivative, theoretical value, 6.96%; found 6.98%; leucine derivative, theoretical value, 6.96%; found 6.95%.

The fate of such amino acids as serine, threonine, histidine, lysine and arginine in this preparation and distillation procedure has not been determined. The occurrence of traces of all of them in the various preliminary distillations seems to indicate that they may be capable of distillation but that most of them are lost in the preparative method described.

The specific rotations of alanine, valine and leucine obtained in crystalline form from pure fractions of their derivatives were determined to indicate the amount of racemization that occurred dur-

ing the over-all preparation and distillation of the derivatives. For alanine, a specific rotation of 6.1 in 2 *N* hydrochloric acid was obtained. This is 42% of the expected value,<sup>10</sup> and indicates that 71% of the material was still in the L-form and 29% had been transformed to the D-form. For valine, a specific rotation of 22.8 in 2 *N* hydrochloric acid was obtained. This is 84% of the expected value,<sup>11</sup> and indicates that 92% of the material was still in the L-form and 8% has been converted to the D-form. Leucine had a specific rotation of 9.9 in 6 *N* hydrochloric acid. This is 65% of the expected value,<sup>11</sup> and indicates that 83% of the material was still in the L-form and 17% had been converted to the D-form. These small conversions to the D-form make it appear probable that the process could be made to yield the L-forms almost exclusively.

Most proteins contain an appreciable amount of glutamic acid, and therefore, the higher boiling fractions have a tendency to be predominantly

(10) P. J. Fodor, V. E. Price and J. P. Greenstein, *J. Biol. Chem.*, **178**, 503 (1949).

(11) M. A. Nyman and R. M. Herbst, *J. Org. Chem.*, **15**, 108 (1950).

pyrrolidone carboxylic acid ethyl ester, and the relative proportions of the other high boiling esters are small. We have, therefore, been unable to accumulate sufficient volumes of these higher boiling esters to permit an efficient fractionation study of them. A promising source of amino acids containing only small amounts of glutamic acid is the dried amino cakes obtained as by-products of the glutamic acid industry. Experiments with them have produced large quantities of the mono-amino-mono-carboxylic acid derivatives.

For large-scale production of amino acids by this method, preliminary separation of the low-boiling and high-boiling materials in a stripping column should be practical, because there is a 15 to 20° difference in the boiling points between these two groups of derivatives.

Further work on the preparation of the mixed acetylated amino acid ethyl esters may increase the yield of these materials. Distillation of these derivatives would then be useful for producing the naturally occurring amino acids in large quantities.

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## The Amino Acid Compositions of Insulins Isolated from Beef, Pork and Sheep Glands

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Samples of beef, pork and sheep insulin which had previously been purified by countercurrent distribution have been analyzed for their amino acid contents. It has been found that the main components of the three insulins differ in their contents of threonine, serine, alanine, glycine, valine and isoleucine. The A and B components of beef insulin appear to differ only by one amide group.

Previous publications from this Laboratory indicate that the major component of beef insulin behaves as a single solute when studied by countercurrent distribution and that it has a molecular weight of the order of 6,000.<sup>1,2</sup> However, a molecular weight of 12,000 has been indicated by several physical methods.<sup>3</sup> Either molecular weight would be possible with the amino acid formula for insulin which Sanger has derived from his extensive structural studies,<sup>4</sup> but, on the other hand, the published figures for the amino acid composition of insulin<sup>5</sup> are in disagreement with his formula as far as certain residues are concerned. Several of the values also are incompatible with a molecular weight as low as 6,000. However, the analytical figures from the different laboratories show considerable discrepancies when compared with each other and give good reason to suspect either errors in the analyses or the use of samples which were not sufficiently homogeneous. We have, therefore, re-examined the quantitative amino acid composi-

tion of beef insulin in the hope that the major component isolated by countercurrent distribution would give an analytical result for every amino acid which would be fully compatible with the lower molecular weight indicated by the method of partial substitution and with the number of residues required by the peptide sequence proposed by Sanger.

Distribution studies with different insulin preparations<sup>1</sup> always revealed a major component (A) and varying amounts of a second component (B) present in smaller amount. The smaller component was found to have physical, chemical and biological properties very similar to those of the major component. An obvious approach to the question of the differences between these two active components was through comparison of their amino acid contents. Purified B component has accordingly been included in the analytical study. It appeared of considerable interest also to include in the study an investigation of the A components of pork and sheep insulins. Although it has been found that the major components of beef, pork and sheep insulins have very similar if not identical partition ratios in the system 2-butanol/1% aqueous dichloroacetic acid, observations concerning certain species differences have been reported.<sup>6</sup>

(1) E. J. Harfenist and L. C. Craig, *THIS JOURNAL*, **74**, 3083 (1952).

(2) E. J. Harfenist and L. C. Craig, *ibid.*, **74**, 3087 (1952).

(3) P. Doty, M. Gellert and B. Rabinovitch, *ibid.*, **74**, 2065 (1952); H. Gutfreund, *Biochem. J.*, **50**, 564 (1952); J. L. Oncley, E. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952).

(4) (a) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951); (b) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353, 366 (1953).

(5) See G. R. Tristram, *Advances Protein Chem.*, **5**, 83 (1949); G. L. Mills, *Biochem. J.*, **50**, 707 (1952); C. Fromageot, *Cold Spring Harbor Symposia on Quantitative Biol.*, **14**, 49 (1950).

(6) (a) F. Sanger, *Nature*, **164**, 529 (1949); (b) J. Lens and A. Evertzen, *Biochim. Biophys. Acta*, **8**, 332 (1952).