3. A separation of 21 amino acids by two-dimensional ionophoresis is given.

4. A separation of monoiodotyrosine, di-iodotyrosine, tri-iodothyronine and thyroxine from 15 other amino acids and an inorganic iodide ion is described.

5. A separation of 18 *N*-acetyl amino acids by one-dimensional paper ionophoresis followed by one-dimensional chromatography is also given.

The apparatus was originally constructed and developed at the Cereals Research Station, St Albans, Herts, and the author is grateful to Dr T. Moran, C.B.E., Director of the Research Association of British Flour Millers, for his encouragement and to Mr G. G. Grindley for technical assistance. The application of the apparatus to the separation of *N*-acetyl amino acids was part of a research programme supported by the British Empire Cancer Campaign and the author would like to thank Professor F. Dickens, F.R.S., and Professor J. Roberts for their interest, and Mr D. Beale for technical assistance.

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Determination of Amino Acids by Double Isotope-dilution Technique

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Radioactive tracers in the form of labelled reagents are proving more and more useful in the micro-analysis of compounds occurring in biochemical materials. Keston and his co-workers successfully employed *p*-iodobenzenesulphonyl chloride labelled with either ^{131}I or ^{35}S as a general reagent for action with amino acids. The simple form of the quantitative tracer method as originally used by Keston, Udenfriend & Cannon (1946, 1949) with ^{131}I -labelled *p*-iodobenzenesulphonyl chloride requires the complete separation and recovery of the derivatives. This disadvantage was overcome by Keston, Udenfriend & Levy (1947, 1950) by adding a known quantity of the ^{35}S -labelled *p*-iodobenzenesulphonyl amino acid after the preparation of the p - ^{131}I iodobenzenesulphonyl derivatives in the sample to be analysed. In this double-tracer technique, a value for the amount of amino acid initially present could be calculated from a measurement of the $^{35}\text{S}/^{131}\text{I}$ ratio for the purified derivative. This estimate was independent of losses from the point in procedure when the known amount of labelled amino acid derivative was added.

The main disadvantage of applying this method to routine use is the frequent preparation of the *p*-iodobenzenesulphonyl chloride necessitated by

the comparatively short half-lives of the labelling isotopes. It was thought that acetic anhydride labelled with ^{14}C and ^3H would prove more satisfactory as a general reagent in double-isotope-tracer methods. Avivi, Simpson, Tait & Whitehead (1954) employed these reagents for the acylation of the hydroxyl groups of hydrocortisone and aldosterone (electrocortine) in the estimation of these compounds in human peripheral blood. This paper describes the extension of the method to the assay of submicrogram quantities of amino acids in protein hydrolysates.

EXPERIMENTAL

Reagents

[1- ^{14}C]Acetic anhydride. This compound was originally prepared by heating inert acetic anhydride and anhydrous sodium [1- ^{14}C]acetate in a sealed tube at 140° for 4 hr. Under these conditions complete exchange of acetyl groups take place.

This reagent is now obtainable from the Radiochemical Centre, Amersham, at a specific activity of 2 mc/m-mole. A stock solution of the labelled reagent diluted with redistilled inert compound to a specific activity of 40 μC /m-mole was dissolved (5 mg./ml.) in redistilled toluene and stored in a glass-stoppered bottle.

Tritiated acetic anhydride. This compound was prepared from tritiated acetic acid produced by the decarboxylation of malonic acid which had been allowed to exchange with tritiated water (Avivi *et al.* 1954), as follows:

(a) The acetic acid was neutralized to phenolphthalein with 2*N*-NaOH. Water was removed *in vacuo* and assayed for tritium. The residual sodium acetate, made anhydrous by heating *in vacuo* at 200° for 2 hr., was exchanged with inert acetic anhydride as described for the preparation of the ¹⁴C-labelled compound.

(b) For the preparation of a reagent of high specific activity, the tritiated acetic acid was divided into two equal portions. One portion was converted into acetyl chloride by the method of Cox & Turner (1950) and the other portion rendered anhydrous as in (a). The acetyl chloride was distilled *in vacuo* on to the anhydrous sodium acetate and the resulting acetic anhydride was distilled off *in vacuo*.

From 0.5 g. of malonic acid and 0.2 ml. of tritiated water containing 400 mc, a 75% yield of labelled acetic anhydride (specific activity 18 mc/m-mole) was obtained.

A stock solution of tritiated acetic anhydride of specific activity of 9 mc/m-mole in redistilled toluene (5 mg./ml.) was used for general analysis.

***N*-Acetyl amino acids.** Samples of the *N*-acetyl derivatives of naturally occurring amino acids were prepared by the methods of du Vigneaud & Meyer (1932), Synge (1939) and Gordon, Martin & Synge (1943). Stock solutions (1 mg./ml.) were made up in ethanol-aq. 2*N*-NH₃ soln. (1:1, v/v) and stored in glass-stoppered tubes.

¹⁴C-Labelled *N*-acetyl amino acids. These labelled derivatives were mostly prepared by acetylating 10 mg. of the appropriate amino acid with ¹⁴C-labelled acetic anhydride in the manner described for acetylation of the analytical samples. A separate apparatus was reserved for these preparations. The inert *N*-acetyl derivative (100 mg.) was added and the solution passed through a Zeo-Karb 225 column. The effluent and washings were evaporated to dryness *in vacuo* and the material was recrystallized from the appropriate solvent.

***N*-α-[1-¹⁴C]Acetylhistidine and *N*-α-[1-¹⁴C]acetylarginine** were prepared by heating 100 mg. of the L-amino acid with 135 mg. of [1-¹⁴C]acetic anhydride (containing 40 μc) and 300 mg. of acetic acid in a stoppered tube in a boiling-water bath for 2 hr. Water (1 ml.) was added and the mixture evaporated to dryness *in vacuo*. The addition of water and evaporation to dryness was repeated twice. The residue was recrystallized from aqueous acetone.

Stock standard solutions (500 μg./ml.) were made up in ethanol-aq. 2*N*-NH₃ soln. (1:1, v/v) and stored in glass-stoppered tubes in the refrigerator. Portions (0.1 or 0.2 ml.) giving 5000–10 000 counts/min. were used for the determination of recovery factors in the assay experiments.

In all these preparations the DL mixtures of the amino acids were used except in the *N*-acetyl derivatives of glutamic acid, tyrosine, histidine and arginine, when the L-amino acids were used.

Standardization of tritiated acetic anhydride

The equivalent specific activity of the tritiated reagent was given by a determination of the specific activity of a pure sample of hydrocortisone acetate prepared with the labelled acetic anhydride (Avivi *et al.* 1954).

Dry hydrocortisone (2 mg.) was dissolved in 0.1 ml. of pyridine, and 0.05 ml. of the tritiated acetic anhydride was added. The sterol acetate was purified by column-partition chromatography with 15 g. of kieselguhr (Celite 545) and 7.5 ml. of methanol-water (4:1, v/v) as stationary phase packed into columns (1 cm. diam. × 30 cm.). Benzene-light petroleum (b.p. 80–100°) (1:1, v/v) was used as the mobile phase. Fractions (3 ml.) were taken by an automatic-fraction cutter after the appearance of the front, which was detected by a band of Sudan red III dye applied to the column. The tube containing the maximum concentration of sterol acetate (approx. 24 ml. from the front) was evaporated to dryness and the residue dissolved in ethanol. The concentration of this solution was determined by measurement of u.v. light absorption with a Hilger Uvispek instrument at 238 mμ and by measurement of the density at 410 mμ of the colour obtained by the Porter-Silber reaction. Portions of the solution were also evaporated to dryness together with inert steroid in silica boats for combustion and counting.

Preparation of samples for analysis

Standard solutions (1 mg./ml.) of chromatographically pure amino acids were prepared in water or 0.1*N*-HCl, with the addition of a few drops of toluene as preservative. Appropriate portions of these solutions were taken for the preparation of a synthetic mixture having a similar composition to the hydrolysate obtained from the fraction A peptide of oxidized insulin.

A sample of fraction A peptide was prepared from crystalline insulin (obtained from Boots Pure Drug Co. Ltd.) by the method of Sanger (1949). The material was purified by band-paper electrophoresis in 0.05*N*-ammonium acetate (pH 8.5) on Whatman no. 3MM paper.

A sample of purified chymotrypsinogen was kindly given by Dr Stanford Moore of the Rockefeller Institute, New York.

Hydrolysates (72 hr.) of the fraction A peptide, crystalline insulin and chymotrypsinogen were prepared by heating 100 μg. in sealed-capillary tubes with 0.1 ml. of 10*N*-HCl *in vacuo* at 105°. The hydrolysates were made up to 25 ml. with water and suitable portions were taken for acetylation.

Acetylation

The apparatus used for acetylation of the samples is shown in Fig. 1. The sample (1–5 ml.) is placed in tube *A*, which carries a B 14 standard ground-glass joint for subsequent connexion to a high-vacuum manifold and a tap at its base to facilitate transfer of the reaction mixture to the ion-exchange resin column. The tube is cooled in an ice bath. The sample solution is neutralized with 0.1*N*-NaOH and a further 0.2 ml. of the alkali is added. A solution (0.2 ml.) of the tritiated acetic anhydride in toluene is pipetted through *B* into the vaporizer tube *C*, which is heated by vapour from boiling CHCl₃ in flask *D* refluxing from condenser *E*. The stopper at *B* is replaced and a steady stream of nitrogen is passed through the delivery tube *F*, which is closed by a sintered-glass plate. The nitrogen carries the toluene and acetic anhydride vapour through the sample via the delivery tube *G*, which is also closed by a sintered-glass plate. Five further additions of 0.2 ml. of 0.1*N*-NaOH to *A* and 0.2 ml. of the tritiated reagent to *C* are made.

Portions of standard solutions of ^{14}C -labelled acetyl derivatives (approx. 50 μg . and 6000–8000 counts/min.) of the amino acids being assayed are added to the acetylated sample. Tube *A* is removed from the apparatus and connected by the B 14 joint to a high-vacuum manifold and the solution is concentrated until all visible traces of toluene are removed.

Removal of excess of sodium acetate

The solution, adjusted to 5 ml., is passed through a column (1 cm. diam. $\times$ 5 cm.) of Zeo-Karb 225 (particle size 50 μ) in the acid condition. Sodium ions are retained on the resin and the acetyl amino acids together with acetic acid pass into the receiver. Any active compounds remaining on the resin are removed by washing the column with ethanol-aq. 2*N*- NH_3 soln. (1:1, v/v) until the darker-coloured front on the resin reaches the bottom of the column. The eluate is evaporated to dryness *in vacuo*. Three additions of 0.5 ml. of 10% (v/v) acetic acid, followed by evaporation to dryness, are made to dilute isotopically traces of active free acetate remaining in the residue. The residue is dissolved in 0.1 ml. of ethanol-aq. 2*N*- NH_3 soln.

Fig. 1. Acetylation apparatus. Lettering is that used in the text.

(1:1, v/v) and applied to Whatman no. 2 filter paper for chromatographic or electrophoretic separation.

Separation of *N*-acetyl amino acids

Solvent systems. (a) Butanol-methanol-aq. 2*N*- NH_3 soln. (4:1:5, by vol.) (two phases); (b) benzene-ethanol-liquid phenol (B.P.) (1:1:5.5, by vol.) (one phase); (c) ethyl acetate-methanol-aq. 2*N*- NH_3 soln. (5:1.5:1.9, by vol.) (two phases). All solvents are used without redistillation.

Chromatography. The *N*-acetyl amino acids are separated by two-dimensional paper chromatography with the appropriate pairs of the solvent systems or by paper chromatography in one dimension followed by paper ionophoresis in 0.05*N*-ethylamine acetate buffer, pH 9, in the second dimension (Whitehead, 1958).

All chromatography is carried out by upward irrigation. The upper organic layer is used as the mobile phase in solvent mixtures (a) and (c) and no equilibration is found necessary in any of the systems. The tanks for use of solvents (a) and (b) are kept in a thermostatically controlled box at 25° and those for solvent (c) in a temperature-controlled room at 18°. In two-dimensional chromatographic separations solvent (b) gives the best results if used as the second solvent.

The completed chromatograms are dried in a current of warm air, followed by heating in the oven at 100° for 15 min. The separated *N*-acetyl amino acids are shown as yellow spots on a blue background by dipping the paper into a solution of bromocresol green in acetone (100 mg./l.) which has been rendered blue by the addition of a drop of morpholine. When histidine or arginine is being assayed the chromatogram should be dipped in neutral indicator solution, when the *N*-acetyl derivatives of these compounds show as blue spots on a yellow background. The spots on the chromatograms should be ringed in pencil immediately after development as rapid fading frequently occurs owing to traces of NH_3 present in the atmosphere of the laboratory.

Measurement of radioactivity

The areas of paper (approx. 1–2 cm.²) containing the labelled *N*-acetyl derivatives of the amino acids being assayed are cut out from the developed chromatograms and burnt in a stream of O_2 by the empty combustion-tube method (Ingram, 1948). The CO_2 and water are trapped in liquid N_2 (Glascock, 1952). The water is held in the traps with baths of solid CO_2 -ethanol, the CO_2 frozen into gas counters and the ^{14}C content measured by the technique of Brown & Miller (1947) with 2 cm. Hg pressure of CS_2 and 20 cm. Hg pressure of CO_2 .

The water is transferred to a zinc-filled H_2 generator (Avivi *et al.* 1954) heated at 400° and the H_2 produced pumped by an automatic Toepler pump into gas counters. The ^3H content was measured by the technique of Faltings (1953) with a pressure of 2 mm. Hg of toluene vapour and 25 cm. Hg of H_2 .

The counters used in both assays were of 100 ml. capacity with graphite cathodes of 1 in. diam. A slight memory effect (20–30 counts/min.) occurred with the counters used for tritium counting after about 30 analyses. After the grease had been cleaned from the tap and the counter left open to the air for 48 hr. in the oven at 100°, this contamination could be removed.

RESULTS

Figs. 2 and 3 show the two-dimensional chromatograms obtained with mixtures of *N*-acetyl amino acids when run in butanol-methanol-aq. NH_3 soln., benzene-ethanol-phenol and ethyl acetate-methanol-aq. NH_3 soln. The abbreviations for the names of the amino acids shown in the figures are those used by Brand & Edsall (1947). The chromatographic pattern obtained with a given pair of solvents is constant, although the actual R_F values may vary considerably. If a number of markers are run simultaneously this variation presents no difficulty. Traces of tritiated acetate remaining in the sample when applied to the paper may be found on the completed chromatogram extending over a wide area but usually restricted to within the areas indicated by the dotted lines in the figures. High results may be obtained in consequence of this 'paper' blank in the assay of glutamic acid, serine and threonine. Occasionally, the contaminated area may spread to include glycine, alanine and tyrosine. These amino acids may be determined by separating the *N*-acetyl derivatives by chromatography, followed by paper electrophoresis when the active impurity is removed from the vital area of the paper. Aspartic and glutamic acids are also determined in this way, as the *N*-acetyl derivatives are not completely separated by chromatography alone. No satisfactory method has yet been found for the separation of *N*-acetyl-histidine and *N*-acetylarginine or the separation of the *N*-acetyl derivatives of the leucine isomers.

Fig. 2. Two-dimensional chromatogram of *N*-acetyl amino acids run in benzene-ethanol-phenol-water (vertical axis) and butanol-methanol-aq. NH_3 soln. (horizontal axis). The broken line indicates the area giving high paper blanks owing to tritiated acetate ion. The abbreviations for the names of the amino acids are those used by Brand & Edsall (1947).

Fig. 3. Two-dimensional chromatogram of *N*-acetyl amino acids run in benzene-ethanol-phenol-water (vertical axis) and ethyl acetate-methanol-aq. NH_3 soln. (horizontal axis). The broken line indicates the area giving high paper blanks owing to tritiated acetate ion.

anol-aq. NH_3 soln. The abbreviations for the names of the amino acids shown in the figures are those used by Brand & Edsall (1947). The chromatographic pattern obtained with a given pair of solvents is constant, although the actual R_F values may vary considerably. If a number of markers are run simultaneously this variation presents no difficulty. Traces of tritiated acetate remaining in the sample when applied to the paper may be found on the completed chromatogram extending over a wide area but usually restricted to within the areas indicated by the dotted lines in the figures. High results may be obtained in consequence of this 'paper' blank in the assay of glutamic acid, serine and threonine. Occasionally, the contaminated area may spread to include glycine, alanine and tyrosine. These amino acids may be determined by separating the *N*-acetyl derivatives by chromatography, followed by paper electrophoresis when the active impurity is removed from the vital area of the paper. Aspartic and glutamic acids are also determined in this way, as the *N*-acetyl derivatives are not completely separated by chromatography alone. No satisfactory method has yet been found for the separation of *N*-acetyl-histidine and *N*-acetylarginine or the separation of the *N*-acetyl derivatives of the leucine isomers.

Table 1 shows the results obtained in an analyses of a synthetic mixture containing a total weight of 1.6 μg . of nine amino acids. The reproducibility of the method is shown in Table 2, where the results of several separate analyses of the synthetic mixture made for leucine, glycine and glutamic acid content are given. In general the method appears to give values with an accuracy of 5%. The analysis of mixture I was done before the paper-electrophoretic separation of the *N*-acetyl amino acids was developed, and the high results obtained for tyrosine and glutamic acid were due to high paper blanks caused by traces of tritiated acetate ion. The high result for valine was due to some active impurity in the ^{14}C standard acetyl derivative which was subsequently removed by recrystallization. Normal values for the recovery of valine and tyrosine were found (Table 3) in the analysis of mixture II (containing the same amino acids as mixture I but in different proportions and analysed only for the four amino acids given in Table 3). The separation in this case was done by the chromatographic-electrophoretic technique.

One of the main sources of error in the isotope-dilution technique is caused by the random nature of radioactive disintegration. The probability of the observed counts being the correct value is dependent on the total number of counts recorded. In the method described here the final result depends on three activity measurements, namely the added ^{14}C -labelled acetyl amino acid count and

the ^{14}C and ^3H activities of the paper-chromatogram spot. The combined counting errors for each amino acid determination in the synthetic mixtures are given in Tables 1 and 3.

The analyses of 72 hr. hydrolysates from 2 $\mu\text{g.}$ of the fraction A peptide of oxidized insulin, whole insulin and chymotrypsinogen for a number of amino acid residues are given in Table 4. Good general agreement was obtained between the experimental figures and the values calculated from formulae (Sanger, 1953; Ryle, Sanger, Smith & Kitai, 1955) for the fraction A peptide and whole insulin. Similar values to those obtained by other workers using different methods of analysis were found for chymotrypsinogen. The high value obtained for glutamic acid in the analysis of fraction A peptide may include a substantial portion of the glutamine residues present (2 residues/mol.). The glutamine present in whole insulin would appear to have been destroyed in the hydrolysis, as the glutamic acid value for this protein is close to the calculated figure. The asparagine present in insulin has been estimated as

aspartic acid. The amount of aspartic acid appearing in the fraction A hydrolysate was not determined.

DISCUSSION

The sensitivity of any microanalytical method is limited by the amount of blank or background material in the final measurement. In the tracer technique only those impurities which react with the labelled reagent and are subsequently not separated from the required derivative can contribute to blank values. Thus in contrast with methods involving inert reagents it is unnecessary to use highly purified solvents or chemicals in the procedures following the preparation of the labelled derivative. The sensitivity of the tracer technique is also limited by the specific activity of the labelled reagent. The tritiated acetic anhydride used in the analysis of the protein hydrolysates had an equivalent activity of 4.45 $\mu\text{C}/\mu\text{equiv.}$, i.e. 1 $\mu\text{g.}$ of acetyl amino acid gives, under our conditions, 25 000–50 000 counts/min. It can be assumed that the ultimate practical sensitivity is the amount of

Table 1. *Analysis of synthetic mixture I*

Amino acids in mixture	Amount added ($\mu\text{g.}$)	Amount found ($\mu\text{g.}$)	Diff. (%)	Standard counting error (%)	Recovery of ^{14}C counts added (%)
Leucine	0.166	0.165	-0.6	± 1.8	16
Valine	0.221	0.240	+8.6	± 1.6	14
Alanine	0.188	0.186	-1.0	± 2.1	22
Tyrosine	0.135	0.150	+11.1	± 3.0	14
Glycine	0.247	0.236	-3.6	± 1.7	9
Serine	0.131	0.140	+6.8	± 1.5	26
Aspartic acid	0.133	0.139	+4.5	± 1.7	22
Glutamic acid	0.205	0.234	+12.0	± 1.9	14
Cysteic acid	0.207	0.197	-4.9	± 1.7	9

Table 2. *Reproducibility of analysis of synthetic mixture I for three amino acids*

Amino acid	Amount added ($\mu\text{g.}$)	Amount found	
		($\mu\text{g.}$)	Mean $\pm$ s.d. ($\mu\text{g.}$) (%)
Leucine	0.166	0.172, 0.165 0.166, 0.183 0.172, 0.170	0.171 ± 3.4
Glycine	0.247	0.264, 0.231 0.236, 0.234	0.241 ± 5.3
Glutamic acid	0.205	0.236, 0.234 0.232	0.234 ± 1.4

Table 3. *Analysis of synthetic mixture II*

Amino acids in mixture	Amount added ($\mu\text{g.}$)	Amount found ($\mu\text{g.}$)	Diff. (%)	Standard counting error (%)
Leucine	0.663	0.678	+2.3	± 1.7
Valine	0.299	0.295	-1.3	± 1.0
Alanine	0.128	0.124	-3.0	± 1.8
Tyrosine	0.524	0.497	-5.2	± 2.5

Table 4. *Analyses of amino acid residues in fraction A peptide of oxidized insulin, insulin and chymotrypsinogen*

(a) Composition was calculated from formula (Sanger, 1953). (b) Experimental values were obtained by alternative methods (Tristram, 1949). (c) Composition was calculated from formula (Ryle, Sanger, Smith & Kitai, 1955). (d) Values were obtained by microbiological assay (Lewis, Snell, Hirschmann & Fraenkel-Conrat, 1950). (e) Values were obtained by column chromatography (Cohen & Wilcox, 1954).

Fraction A peptide of oxidized insulin				
Amino acid	Amount found (μg.)	Amount found (g./100 g. of protein)	Calc. amount present (a) (g./100 g. of protein)	
Leucine	0.334	16.7	16.0	
Valine	0.178	8.9	9.5	
Alanine	0.068	3.4	3.6	
Tyrosine	0.310	15.5	14.7	
Glycine	0.078	3.9	3.1	
Serine	0.178	8.9	8.6	
Asparagine	—	—	10.7	
Glutamine	—	—	11.6	
Glutamic acid	0.417	20.7*	11.7	
Cysteic acid	0.584	29.2	27.5	

Whole insulin				
Amino acid	Amount found (μg.)	Amount found (g./100 g. of protein)	Amount found (b) (g./100 g. of protein)	Calc. amount present (c) (g./100 g. of protein)
Leucine	0.322	16.1	16.0	16.0
Valine	0.240	12.0	7.8	10.2
Alanine	0.098	4.9	4.5	4.7
Tyrosine	0.266	13.3	13.0	12.6
Glycine	0.106	5.3	4.3	5.2
Serine	0.114	5.7	5.2	5.5
Asparagine	—	—	—	6.9
Aspartic acid	0.126	6.3	6.8	—
Glutamine	—	—	—	7.6
Glutamic acid	0.212	10.6	18.6	10.3
Phenylalanine	0.180	9.0	8.1	8.7
Proline	0.046	2.3	2.5	2.0
Lysine	0.054	2.7	2.5	2.6
Threonine	0.042	2.1	2.1	2.1

Chymotrypsinogen				
Amino acid	Amount found (μg.)	Amount found (g./100 g. of protein)	Amount found (d) (g./100 g. of protein)	Amount found (e) (g./100 g. of protein)
Leucine	0.266	13.3	13.8	14.1
Valine	0.234	11.7	10.3	9.9
Alanine	0.152	7.6	5.8	7.3
Tyrosine	0.054	2.7	2.7	2.8
Glycine	0.140	7.0	6.3	6.6
Serine	0.204	10.2	10.9	11.9
Aspartic acid	0.212	10.6	10.9	11.0
Glutamic acid	0.126	6.3	7.4	6.5
Phenylalanine	0.086	4.3	3.8	4.1
Proline	0.068	3.4	3.8	3.8
Lysine	0.146	7.3	7.7	7.6
Threonine	0.204	10.2	10.7	10.4

* This value probably includes glutamine (see text).

amino acid derivative which has an activity equal to the background count (50 counts/min.). With the tritiated acetic anhydride used in this work the value would be 0.001–0.002 μg .

An advantage of the tracer method is the independence of results on losses in procedures following the addition of the labelled amino acid derivative, provided that enough derivative remains for the final counting. In the present method these losses amount to 15–20% on the Zeo-Karb 225 column and 30–40% in each chromatographic separation. Final recoveries varied between about 10 and 25%, according to the amino acid, and the values obtained in the analysis of a synthetic mixture are given in Table 1. Other methods depend on a series of control experiments for an estimate of recovery and this estimate is generally an average value. The automatic correction for losses in the double-tracer method allows the use of highly effective but wasteful purification procedures.

Although the tracer method does not demand the quantitative recovery of the labelled derivative, the reaction with the labelled reagent must go to completion and the separated compound must be isotopically pure. The purity of the derivative can be tested, if necessary, in every estimation by a survey of the isotope ratios obtained in the final separation procedure. With a paper chromatogram the appropriate spot may be cut into two or more portions and each portion assayed for radioactivity separately. If the material is pure, the ratio of ^{14}C to ^3H for each determination will be constant.

The results obtained in this work indicate that the method is often capable of an accuracy of 2–3% and usually not more than 5%. With improved techniques of separation an even higher order of accuracy should be achieved. Tristram (1946) has pointed out that, when the values are required for the estimation of the number of residues of amino acids in the protein molecule, the maximum permitted variation is $\pm 0.4/n$, where n is the number of residues of the amino acid in question. It would appear that the tracer method should give the required accuracy on as little as 2 μg . of protein in the majority of experiments (i.e. when $n \geq 10$ –20).

SUMMARY

1. A double isotope-dilution method of analysis for the determination of amino acids in protein hydrolysates obtained from small samples of

proteins with acetic anhydride labelled with ^{14}C and ^3H is described.

2. The method has been applied to synthetic mixtures of amino acids and to the 72 hr. hydrolysates obtained from 2 μg . of two proteins of known composition, namely the fraction A peptide of oxidized insulin and whole insulin.

3. Values are given for the analysis of chymotrypsinogen and these are compared with the results obtained by microbiological assay and by column chromatography.

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