Determination of Monoamino Monocarboxylic Acids by Quantitative Paper Chromatography

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Many methods for chromatographic separation of amino acids are now available, but their subsequent determination, generally employing ninhydrin, often leaves much to be desired. It was felt, therefore, that there was a need for a simple method of analysis based on the convenient paper technique and employing some means of determination less fickle than ninhydrin colorimetry.

The method now presented involves an initial one-dimensional separation of amino acids in considerable quantity, using bands rather than spots. The problem of accurate marking of these bands, with little space between them and often having irregular fronts, has been solved by spraying with an indicator solution of bromothymol blue, formaldehyde and alkali, in ethanol (Kemble & Macpherson, 1952). This restricts the range of solvents to those neutral in character, but several such are known to give useful separations of amino acids. Methods of determination were then investigated. Measurement of carbon dioxide following oxidation with ninhydrin is a most specific and accurate method (Van Slyke, Hamilton, McFadyen & Dillon, 1941), but note was taken, in the same paper, of the use of chloramine-T for decarboxylation at low temperatures. Cohen (1940) has measured the evolution of carbon dioxide by chloramine-T in a Warburg respirometer. As numerous determinations can be performed simultaneously, this apparatus was adopted for the present work. A 9 in. wide strip of Whatman 3MM paper will separate at least 2 mg. of each amino acid, sufficient to give a maximum reading on the manometer.

Recoveries of carbon dioxide from amino acids obtained by Krebs (quoted by Cohen, 1940) are significantly higher than the more nearly theoretical values reported by Van Slyke *et al.* (1941). Preliminary work showed that yields of carbon dioxide following direct oxidation with chloramine-T were in accord with the results of Krebs, while yields obtained from a sprayed chromatogram agreed with those of Van Slyke. This difference was found to be due to the suppression by the formaldehyde of the indicator spray of interference due to ammonia, and all recovery factors (moles CO_2 /mole amino acid) have been determined in the presence of a standard amount of formaldehyde.

Work has been confined in the present instance to the monoamino monocarboxylic acids, which can be isolated readily by ionophoresis in a three-compartment cell.

METHODS

Chloramine-T reaction

Standard solutions of amino acids. DL-Amino acids were used with the exception of glycine, L-hydroxyproline and L-tyrosine. In all cases paper chromatograms showed no ninhydrin-reacting impurities to be present, and micro-Kjeldahl determinations showed the nitrogen contents to be 99–100% of the theoretical value.

Standard aqueous solutions of the amino acids were prepared of strength 1–3 mg./ml.

Warburg constant volume respirometer. The temperature of the bath was maintained at 30° and the manometer fluid was prepared as described by Krebs (1951).

For each set of determinations a thermobarometer was used in addition to the manometer for determining the chloramine-T blank and no more than three manometers were read in conjunction with the thermobarometer at any one time. Thus errors due to minor fluctuations of temperature and pressure are avoided.

Determination of recovery factors. The recovery of CO_2 from amino acids was independent of the amount of formaldehyde present over the range 0.1–0.2 ml. formalin (Table 1) and was almost independent of the amino acid concentration. All recovery factors, therefore, were established in presence of 0.15 ml. commercial formalin (37–41% (w/v) aqueous formaldehyde), and the Warburg flasks were prepared by adding a measured quantity of amino acid solution, 0.4 ml. buffer (8.24 g. trisodium citrate and 76.6 g. citric acid dissolved in 250 ml. water, pH 2.5), 0.15 ml. formalin, and water up to a final volume of 2.5 ml. 0.5 ml. aqueous chloramine-T solution (12%, w/v) was added to the side arm.

Table 1. *Moles of gas, measured as CO_2 , evolved from one mole of amino acid by chloramine-T in the presence of varying amounts of formaldehyde at pH 2.5 (see text)*

Amino acid	Quantity of formalin (ml.)					
	0	0.05	0.10	0.15	0.20	0.50
Alanine	1.10	0.99	0.98	0.99	0.99	1.00
Glycine	1.26	0.99	0.99	0.96	0.98	0.90
Leucine	1.08	1.01	1.00	1.02	1.00	0.98
Phenylalanine	1.04	0.99	0.93	0.93	0.92	—
Valine	1.09	1.01	1.03	1.03	1.00	0.97

Table 2. Moles CO₂/mole amino acid evolved by chloramine-T for various amino acid concentrations at pH 2.5 in presence of 0.15 ml. formalin (see text)

Amino acid	Wt. of amino acid (mg.)					
	2.19	1.42	1.10	0.81	0.73	0.40
Serine	—	0.951 ± 0.004	—	0.925 ± 0.012	—	0.897 ± 0.011
Threonine	—	0.918 ± 0.009	—	0.891 ± 0.011	—	0.877 ± 0.018
Valine	1.02 ± 0.01	—	1.03 ± 0.01	—	1.00 ± 0.01	—

Table 3. Recovery factors (moles CO₂/mole amino acid) for individual amino acids in the reaction with chloramine-T at pH 2.5 (see text)

Amino acid	Weight range investigated (mg.)	Moles CO ₂ /mole amino acid		Mean variation	No. of determinations
		Van Slyke <i>et al.</i> (1941)	Present work		
Alanine	1.52-0.46	1.03	0.997	0.006	5
Glycine	1.02-0.26	0.972	0.969	0.006	5
Hydroxyproline	2.54-0.51	—	0.971	0.011	7
Isoleucine	1.91-0.38	—	1.021	0.008	5
Leucine	1.98-0.40	1.017	1.018	0.007	5
Methionine	1.62-0.65	—	0.864	0.009	10
Phenylalanine	2.05-0.51	0.946	0.931	0.006	5
Proline	1.68-0.50	—	0.904	0.007	6
Serine	1.42-0.40	1.028	0.951-0.901	0.004-0.011	3 × 5
Threonine	1.38-0.39	1.015	0.918-0.873	0.009-0.008	3 × 5
Tryptophan*	0.77	0.751	0.33	—	1
Tyrosine†	2.67-0.67	1.033	0.980	0.012	5
Valine	1.98-0.40	—	1.023	0.008	5

* The brown precipitate, observed by Van Slyke *et al.* (1941) when chloramine-T was added to tryptophan, was not obtained in the presence of formalin.

† Solution made up in citrate buffer (pH 2.5).

Table 4. Evolution of gas, measured as CO₂, in reaction between 0.585 mg. NH₃ and chloramine-T in 20 min. (see text)

	pH 2.5		pH 4.7*	
	No formalin	Formalin (0.2 ml.)	No formalin	Formalin (0.2 ml.)
Gas evolved (μl. at s.t.p.)	484.3	15.6	650.1	244.1

* 0.4 ml. buffer (35.3 g. trisodium citrate and 16.8 g. citric acid dissolved in 150 ml. water) was used.

With the exception of proline and hydroxyproline, when 100 min. was needed, gas evolution was complete within 20 min. and final manometer readings were taken accordingly.

For each amino acid at least five determinations were made on different quantities of standard solution, and the yield of gas was found to be independent of amino acid concentration except for serine and threonine. With these the variation amounted to little more than 5% over the range useful for estimation (Table 2).

The appropriate recovery factors listed in Table 3 are applied for all calculations made throughout the remainder of this paper except in the case of tryptophan, where the factor was found to be low and irregular.

Reaction between chloramine-T and substances other than α-amino acids. Chloramine-T is known to form nitrogen from ammonia (Cohen, 1940) and this reaction was investigated. The evolution of gas from ammonia was almost completely inhibited by formaldehyde at pH 2.5 (Table 4).

Primary and secondary amines also evolve gas with chloramine-T but will be removed from the neutral amino acids during ionophoresis.

Under the conditions of analysis, tertiary amines, pyridine, urea, γ-aminobutyric acid and fructose gave no evolution of gas with chloramine-T.

No observations have been made on peptides but at the higher temperature of 45° Van Slyke *et al.* (1941) reported values up to 0.075 moles CO₂/mole for several dipeptides.

Analysis of made-up mixtures

Chromatography tanks. These consisted of glass jars 21 in. deep, of area 11.5 × 7.5 in. and having a glass cover. The troughs were Alkathene tubes (1.5 in. bore) with a 0.8 in. wide slit cut to within 0.5 in. of the ends. They were held in position by pressure of the rubber bungs sealing each end against the walls of the jar.

Preparation of papers. Whatman 3 MM paper 9 in. wide was used with 18.5 in. from the starting line to the bottom of the strip. A margin was ruled down each side, 0.5 and 0.7 in. from the respective edges.

Chromatograms run with propanol:water, when sprayed with ninhydrin, exhibited pinkish 'beards' in front of the spots; the inclusion of cupron in the solvent (Consden, Gordon & Martin, 1944), and even washing papers with an ethanolic solution of cupron before use did not entirely prevent the condition. The following treatment, however, proved successful. A solution of 8-hydroxyquinoline (30 mg. in 1 ml. acetone) was applied along the intended starting line and the paper was then eluted chromatographically with acetone until the reagent colour was

washed from the bottom of the sheet. This took 3–4 hr., and after drying at room temp. the paper was ready for use.

The solution for analysis, containing 1–2 mg. α -amino N and of volume preferably not greater than 2 ml., was applied as a band along the starting line using an 'Aglar' micrometer syringe with a glass needle (Burroughs, Wellcome and Co. Ltd., London). The band was kept well within the limits defined by the ruled margins. After each addition most of the solvent was driven off, using warm air from a hair drier, before more solution was applied. The band was not allowed to exceed 0.5 in. in width. Two identical papers were prepared, to be developed in the solvents described below. They were folded 1.8 in. above the starting line before insertion into the trough and held in position by a glass rod in the bottom of the trough.

Solvents. Many solvents have been described in the literature for the separation of amino acids but, bearing in mind the need for a neutral solvent, the mixture propanol: water (80:20 by vol.) has proved the most useful of those tested.

On a Whatman 3MM paper 18 in. long the following separation was achieved in 32–36 hr. at room temp.: glycine + serine; threonine + hydroxyproline; alanine; proline + tyrosine + tryptophan; valine + methionine; phenylalanine; leucine + isoleucine (order of increasing R_F).

Investigations were carried out to try to find another solvent mixture of different properties in which a second chromatogram could be run and used in conjunction with the one obtained from propanol: water. The most useful of the mixtures tried was isobutanol: methyl ethyl ketone: water (70:50:30 by vol.) which gave the following separation on an 18 in. strip of Whatman 3MM paper in 40–44 hr. at room temp.: glycine + serine; threonine + hydroxyproline + alanine; proline; valine; methionine + tyrosine; isoleucine; leucine partially separated from phenylalanine and tryptophan (order of increasing R_F).

Detection and elution of amino acid bands. After the two chromatograms have run for the requisite time they are removed from the tanks and hung in the laboratory for some minutes. They are not allowed to become quite dry, however, as the indicator spray seems most effective if the paper is still slightly moist with solvent.

The indicator spray, as previously described (Kemble & Macpherson, 1952), has had its formalin content decreased in order that the contribution of the spray to the amount of formaldehyde in the flasks before analysis shall be negligible. A 1% (v/v) content of formalin was found to be sufficient to allow the spray to function satisfactorily. Thus the spray consists of 20 ml. 0.15% (w/v) solution of bromothymol blue in 95% (v/v) aqueous ethanol, 0.2 ml. formalin and 0.2 ml. 20% (w/v) aqueous KOH. The optimum content of the last constituent has been found to vary with different batches of paper.

Using an apparatus giving a fine mist, the chromatogram is sprayed from the top down and, as the position of each band of amino acid is made apparent, it is marked with a ball-point pen before spraying is continued.

When the chromatogram has dried, the strips containing the amino acid are cut out for elution. The 0.7 in. margin is cut in the shape of a V leaving a tail 0.2 in. long and 0.1 in. wide at its point. A fold is made along the 0.5 in. margin and the fold hung over the lip of a small, brass trough and held in position by a microscope slide. The trough, which can be adjusted for height, can accommodate 10–12 strips, which draw water out of the trough and down the paper in

the manner of a chromatogram. The water finally collects in the paper tail at the bottom of the strip and drips off into a tared Warburg flask or a 5 ml. graduated flask suitably placed beneath. The whole apparatus is covered by an inverted glass tank to prevent excessive evaporation of water during elution.

It has been found that when all the indicator has been washed from the paper (about 2 ml. water), then all the amino acid will also have been eluted.

Estimation. After elution the Warburg flasks are weighed to ascertain the volume of the contents.

The eluate from the (threonine + hydroxyproline + alanine) strip of the isobutanol chromatogram is used to determine whether or not hydroxyproline is present. This solution is shaken with 1.0 ml. butanol and the butanol layer, which will now contain the indicator, is discarded. A test for hydroxyproline is carried out on the aqueous layer after the method of Neuman & Logan (1950), using *p*-dimethylaminobenzaldehyde. If hydroxyproline is absent then the eluate from the threonine strip of the propanol: water chromatogram is directly oxidized with chloramine-T.

Should the volume of solution be greater than 2.0 ml. after elution, then the flask constant can be adjusted by use of the equation: $K_f = K - 0.0236\Delta V$, where K is the constant established for a total final vol. of 3 ml. and ΔV ml. is the vol. of eluate in excess of 2.00 ml. This equation also shows that a balance weighing to 0.1 g. is sufficiently accurate to weigh the flask and flask contents since, assuming an average value of 1.700 for K , an error of 0.1 g. in the weight of the flask contents implies a negligible error (0.1%) in the subsequent analysis.

If the strip has been eluted into a 5 ml. flask, as always with glycine + serine and threonine + hydroxyproline mixtures, then a known sample is transferred to a Warburg flask.

Subsequent treatment is exactly as has been described for standard amino acid solutions.

Blanks. The ammonia content of the paper, which has caused so much trouble with ninhydrin-colorimetric determinations, presents no problem in these estimations. The amount of ammonia which can be washed from a sprayed strip of 3MM paper 9 × 2 in. has never been found to exceed 0.05 mg. and thus can be neglected as a contributor towards total gas evolution (Table 4).

There remains the possibility of other contaminants reacting with chloramine-T, but during a series of determinations made on blank chromatograms run in neutral solvents the volume of gas evolved from the eluates never exceeded that of the chloramine-T blank by more than 2 μ l. However, it is necessary to distil commercial isobutanol from acid solution before use.

Thus the only blank determination which has to be made is on the chloramine-T solution.

Mutual effect of two amino acids on respective CO_2 evolutions. Since no known solvent will completely separate all the amino acids, it is sometimes necessary to estimate the total evolution of gas from a mixture. For example, using propanol: water (80:20, v/v) as solvent, serine and glycine cannot be separated. However, determinations carried out for weight ratios ranging from 4:1 to 1:4 showed that the amount of gas evolved by each amino acid is not affected by the presence of the other.

Glycine + serine and hydroxyproline + threonine bands. As has been stated, these pairs of amino acids are not separated by the solvents employed, and additional treatment is necessary for their determination.

Table 5. *Recovery of glycine following periodate oxidation of glycine + serine mixtures (see text)*

Time in desiccator	Mixture (mg.)		Gas evolved (μ l. at s.t.p.)	Recovery (%)
	Serine	Glycine		
30 min.	1.35	—	2.1	—
	1.35	0.25	73.6	99.7
	1.35	0.51	150.8	102.2
	0.54	0.51	143.3	97.1
	—	0.51	147.1	99.7
Overnight	1.35	—	3.6	—
	1.35	0.25	55	74.5
	1.35	0.51	134.8	91.3
	—	0.51	149.8	101.5

Table 6. *Recovery of hydroxyproline following periodate oxidation of threonine (see text)*

Time of periodate oxidation (min.)	Mixture (mg.)		Gas evolved (μ l. at s.t.p.)	Recovery (%)
	Threonine	Hydroxyproline		
0.25	1.37	—	4.8	—
0.25	—	2.03	335.3	99.2
1.0	—	2.03	336.1	99.4
5.0	—	2.03	303.5	89.8
10.0	—	2.03	283.0	83.7
0.5	1.37	—	6.5	—
0.5	1.37	2.03	335.2	99.2
0.5	—	2.03	334.8	99.0
0.5	1.97*	—	179.3	—

* From chromatogram.

Table 7. *Recovery of hydroxyproline following removal of formaldehyde and periodate oxidation of threonine (see text)*

Mixture (mg.)		Gas evolved (μ l. at s.t.p.)	Recovery (%)
Threonine	Hydroxyproline		
1.48	—	6	—
0.59	0.51	83.7	98.9
0.59	1.02	166.7	98.5
0.30	0.51	80.7	95.4
—	1.02	167.5	99.0

Serine and threonine are oxidized by a neutral or alkaline solution of periodate to give ammonia, glyoxylic acid and the appropriate aldehyde (Nicolet & Shinn, 1939), and determination of the β -hydroxy α -amino acid content of these mixtures was attempted by measurement of the ammonia formed. This was unsuccessful, low and erratic recoveries being obtained by microdiffusion or by aeration. It has been found possible, however, to destroy the serine or threonine with periodate and determine the remaining amino acid by chloramine-T.

(a) *Glycine + serine mixtures.* A sample of the glycine + serine solution, containing up to 1.2 mg. glycine, is pipetted into a tared Warburg flask and made to 2 ml. with distilled water. 0.2 ml. 2N-NaOH is added, followed by 0.3 ml. 0.5M periodic acid and the flask gently shaken to ensure thorough mixing. After 1 min., 0.1 ml. ethylene glycol is added and mixed by shaking to ensure destruction of excess of periodate. 0.1 ml. conc. H_2SO_4 is now put in the centre

well of the flask which is placed in a vacuum desiccator for 30 min. The acid is then removed from the well and the solution adjusted to pH 7 with 2N- H_2SO_4 , after which the flask is weighed to ascertain the volume of its contents, and the glycine is determined in the usual way with chloramine-T. A blank determination on 2 ml. water with the reagents is carried out simultaneously. When the glycine exceeds 0.3 mg., recoveries of $100 \pm 3\%$ are obtained (Table 5), but it is important that the flask should not remain in the desiccator for more than 30 min., which period is sufficient to remove the ammonia, since it was found that when left overnight, low recoveries of glycine from glycine + serine mixtures occurred (Table 5). Glycine alone is unaffected so the loss must be due to its reaction with one of the oxidation products of serine, presumably glyoxylic acid.

The accuracy of the serine estimation, arrived at by subtracting the glycine value from that obtained for the glycine + serine mixture, depends on the amount present. Where this is greater than 0.4 mg., recoveries are $100 \pm 5\%$.

The procedure described is applicable to the eluate of the glycine + serine band after spraying with the indicatorspray, provided periodate oxidation is performed immediately after elution. When the solutions were kept for some days, the oxidation of serine was incomplete. Experiment showed that this inhibitory effect was due to formaldehyde and that inhibition was markedly less in alkaline than in neutral solutions. The eluate from a sprayed strip is normally slightly alkaline, hence the satisfactory results on immediate periodate oxidation. As a precaution, however, the glycine + serine strip is lightly sprayed with 2N-NaOH before eluting and the eluate oxidized immediately.

(b) *Threonine + hydroxyproline mixtures.* The procedure described for glycine + serine mixtures is also applicable to threonine + hydroxyproline solutions, but the time allowed for periodate oxidation is decreased to 30 sec. as hydroxyproline also seems to be attacked, although much more slowly (Table 6).

When applied to eluates from sprayed chromatograms, the inhibitory effect of formaldehyde on periodate oxidation is much more marked with threonine than with serine and it is not possible to get anything approaching complete oxidation no matter how soon after elution the periodate is added (Table 6, last entry). The following procedure, however, seems to break up the threonine-formaldehyde complex. The Warburg flask containing the threonine + hydroxyproline is made approx. 2N with respect to HCl and allowed to remain on a boiling-water bath until thoroughly dry. If the contents are now dissolved in water and treated with periodate, as described above, the threonine is oxidized completely and hydroxyproline can be determined (Table 7). These experiments indicate a recovery of $100 \pm 5\%$.

The accuracy of the threonine value, determined by difference, will depend on the relative amounts of threonine and hydroxyproline present. Except in gelatin and related products, hydroxyproline is normally present in small amount or is absent altogether. In the latter case, a direct determination of threonine can be made to within 5% and in the former, accuracy will be within 8%.

Analysis of natural products

Ionophoresis. In extracts of natural products the free amino acids will be contaminated with salts which must be removed before chromatography and this can be done by ionophoresis in a three-compartment cell (Macpherson,

1946), the basic and acidic amino acids being removed at the same time.

The extract or protein hydrolysate containing up to 20 mg. N is adjusted to pH 6 with dil. NH_3 and placed in the centre compartment of a three-compartment cell with water in the outside compartments. Direct current is passed and migration of charged substances occurs. As this migration nears completion the current will fall and a minimum value (20–40 mA in the apparatus used) will be reached when migration is complete. The centre-compartment solution, which will contain a large proportion of the monoamino monocarboxylic acids, is removed and the contents of the two outside compartments are mixed, adjusted to pH 6 with dil. NH_3 and again subjected to ionophoresis. This process is repeated.

A mixture of the three centre-compartment solutions will now contain all the monoamino monocarboxylic acids originally present.

Hydrolysis of egg albumin and insulin. The procedure used was as follows. Up to 300 mg. protein were dissolved by warming with 30–40 ml. 10N-HCl (A.R.) and the solution was made up to 50 ml. with 10N-HCl. 1 ml. samples were taken for determinations of total N and the remainder of the solution was made up to 500 ml. 6N-HCl and refluxed for 24 hr. The hydrolysate was taken to dryness under reduced pressure, redissolved and filtered to remove humin. The filtrate was made up to 50 ml. and a sample taken for determination of total N and ammonia N. The remainder was subjected to ionophoresis as described above.

No interference on chromatograms due to cysteine was observed and it is assumed that any cysteine formed during hydrolysis was oxidized to cystine during the preparatory processes (Stein & Moore, 1949).

When tyrosine and tryptophan both occur, then, at present, some other method should be used, e.g. one of the available colorimetric procedures.

The determination of cystine has not been worked out, but as it runs behind glycine in both solvents, it does not interfere with the above scheme. This amino acid, together with cysteine, is discussed below.

RESULTS

Made-up mixtures

The method has been applied to mixtures containing various amounts of some or all of the amino acids listed in Table 3. Two analyses of such a mixture are set out in Table 8, together with a summary of the recoveries experienced in the course of analyses of several such mixtures.

Natural products

Egg albumin. A sample of egg albumin (crystallized) obtained from Armour Laboratories was used. The nitrogen content was 15.45% on a moisture-free basis. Two independent hydrolyses were carried

Summary of scheme of analysis for monoamino acids

Completely separated	Direct estimation after use of periodate	Estimation by difference after periodate oxidation	Estimation by difference
Alanine (P)	Glycine (P and B)	Serine (P and B)	Leucine
Isoleucine (B)	Hydroxyproline (P)	Threonine (P)	Methionine
Phenylalanine (P)			Tyrosine
Proline (B)			(if tryptophan absent)
Valine (B)			
Threonine (P)			
(if hydroxyproline absent)			

(P) Propanol:water (80:20 by vol.).

(B) *iso*Butanol:methyl ethyl ketone:water (70:50:30 by vol.).

Table 8. *Analysis of a made-up mixture of amino acids*

Amino acid	Taken (mg./ml. soln.)	0.5 ml. soln. analysed		1.0 ml. soln. analysed		Range of recoveries for amino acids (N > 0.08 mg.) observed with numerous made-up mixtures
		Gas evolved ($\mu\text{l. at s.t.p.}$)	Recovery (%)	Gas evolved ($\mu\text{l. at s.t.p.}$)	Recovery (%)	
Glycine	1.614	92.2*	98.7	192.0*	102.8	103–97
Serine	1.782	66.0*	97.5	135.5*	96.4	105–95
Threonine	1.236	102.8	97.9	218.5	103.3	105–95
Alanine	1.600	197.1	98.1	390.8	97.3	103–97
Proline	1.352	135.6	100.6	235.8	99.0	103–97
Tyrosine	0.596	41.6	115	70.1	96.9	108–92
Valine	2.372	226.3	97.4	181.5*	97.7	103–97
Methionine	1.228	75.5	94.6	66.5*	104.2	105–90
Phenylalanine	1.680	102.6	96.6	221.5	104.3	105–95
Leucine	1.520	133.2	100.6	280.9	106.2	106–97
Isoleucine	1.360	113.9	95.8	226.1	95.2	99–93
(Leucine + isoleucine)	2.880	247.1	98.4	507	101	103–97

* Gas evolution for 40% of eluate.

out and two analyses made of each hydrolysate (Table 9).

Threonine, in the absence of hydroxyproline, was determined by direct oxidation with chloramine-T. The 'before glycine' value includes that part of the chromatogram between glycine and the starting line and will include any cystine present. An arbitrary carbon dioxide yield of 95% has been assumed for this.

Insulin. A sample of ox insulin (containing zinc)

obtained from Boots Pure Drug Co. Ltd. was used. The nitrogen content was 15.12% on a moisture-free basis. One hydrolysis and two amino acid determinations were made (Table 10). The large tyrosine content caused some precipitation to occur on concentration and this was removed. No tyrosine figure is noted, therefore, but in any case the determination of tyrosine is more accurate when carried out independently on an alkaline hydrolysate of protein.

Table 9. *Monoamino acid composition of egg albumin (values are N as % protein N)*

	1st hydrolysate		2nd hydrolysate		Average	Microbiological assay values	Other values
	1st det.	2nd det.	1st det.	2nd det.			
Glycine	4.34	—	4.08	4.11	—	—	—
Propanol	4.31	—	4.23	4.20	4.21	4.2 ¹	3.85 ⁷
isoButanol	—	—	—	—	—	—	—
Serine*	6.68 (7.46)	—	6.28 (7.02)	6.29 (7.03)	6.47 (7.23)	7.92 ¹ , 7.02 ²	6.92 ⁸
isoButanol	6.63 (7.41)	—	6.56 (7.33)	6.36 (7.11)	—	—	—
Threonine*	3.01 (3.18)	—	3.34 (3.53)	3.22 (3.40)	3.19 (3.37)	3.46 ¹ , 2.7 ³	3.01 ⁸
Hydroxyproline	0	0	0	0	0	—	—
Alanine	—	6.40	6.17	6.41	6.33	7.47 ⁴	6.69 ⁹
Proline	2.84	2.71	2.65	2.85	2.76	6.16 ¹	2.51 ¹⁰
Tyrosine	1.56	—	1.71	1.52	1.60	1.74 ⁵	2.01 ¹⁰
Valine	5.48	5.72	5.56	5.56	5.58	5.31 ³	3.5 ⁹
Methionine†	2.41	1.84	2.45	1.98	2.29	2.44 ³	3.11 ¹¹
Phenylalanine	3.89	4.34	—	3.73	3.99	4.25 ³	4.0 ⁹
Isoleucine	4.31	4.50	4.30	4.22	4.33	4.74 ³	} 10.8 ⁹
Leucine	6.03	—	5.80	6.38	6.07	6.2 ³ , 6.6 ⁸	
'Before glycine'†	0.6	—	0.8	—	0.7	—	—
Total N of monoamino acids	47.5 (micro-Kjeldahl)		47.4 (micro-Kjeldahl)		47.6	—	—
Insol. humin	7.5		7.2		—	—	—
Ammonia (formed on hydrolysis)	9.0		8.7		—	—	—

* Figures in brackets are values corrected for destruction during hydrolysis (Rees, 1946).

† See text.

(1), Sauberlich, Pearce & Bauman (1948); (2), Boyd & Logan (1942); (3), Stokes, Gunness, Dwyer & Caswell (1945); (4), Sauberlich & Bauman (1949); (5), Gunness, Dwyer & Stokes (1946); (6), Ryan & Brand (1944); (7), Stein (1943); (8), Rees (1946); (9), Tristram (1946); (10), Chibnall (1946); (11), Kassell & Brand (1938).

Table 10. *Monoamino acid composition of ox insulin (values are N as % protein N)*

	1st det.	2nd det.	Average	No. of residues/molecule	
				Found*	Reported†
Glycine	6.38	6.30	6.34	8.4	8
Serine†	4.01 (4.42)	4.27 (4.77)	(4.63)	6.1	6
Threonine†	2.21 (2.34)	2.24 (2.37)	(2.36)	3.1	2
Hydroxyproline	Absent		0	0	0
Alanine	4.84	4.97	4.91	6.5	6
Proline	1.55	1.64	1.60	2.1	2
Valine	{ 7.15 6.96§	7.14	7.08	9.5	10
Methionine	Absent		0	0	0
Phenylalanine	3.91	3.99	3.95	5.2	6
Isoleucine	1.40	} 10.1	1.40	1.9	2
Leucine	8.60		8.60	11.4	12
'Before glycine'	5.0	—	5.0	—	—

* Calculated assuming mol.wt. 12000 and N content 15.54% for Zn-free insulin.

† Compiled from Sanger & Tuppy (1951) and Sanger & Thompson (1953).

‡ Bracketed values are corrected for hydrolysis losses (Rees, 1946).

§ Determined on propanol:water chromatogram in absence of methionine.

DISCUSSION

The essential difference between the chloramine-T reaction as performed by Krebs (Cohen, 1940) and Van Slyke *et al.* (1941) is that Krebs, using a Warburg respirometer, was measuring total gas evolved, whereas Van Slyke *et al.*, by first absorbing the carbon dioxide in alkali, were ensuring that only carbon dioxide was measured. A pointer to the reason for Krebs's high recoveries is contained in a footnote to Cohen's paper, where it is stated that ammonium salts interfere with the estimation, giving gaseous nitrogen with chloramine-T. While Dakin (1916) showed that excess of chloramine-T gives a nitrile instead of aldehyde and ammonia, in no case were his yields quantitative. A simple calculation from the data of Table 4 shows that if, at pH 4.7, 10% of the nitrogen of an amino acid should form ammonia instead of nitrile, then this would result in an increase of approximately 10% in the apparent amount of carbon dioxide evolved, and it is significant that glycine, which gives only a trace of hydrocyanic acid (Dakin, 1916), should give the greatest evolution of gas (Table 1).

It is suggested, therefore, that the high results obtained by Krebs are due to the formation in part of ammonia which is then oxidized to gaseous nitrogen. This suggestion is given added weight by the observed effect of formaldehyde on the evolution of gas both from ammonia and from amino acids. At pH 2.5 the amount of gas obtained from ammonia is very greatly diminished by the presence of formaldehyde (Table 4), while the amount of gas evolved by the individual amino acids is also decreased (Table 1).

Ammonia is known to combine with formaldehyde in a variety of ways, but it is surprising to find that this combination is apparently much stronger at pH 2.5 than at pH 4.7.

When any method of analysis is based on a factor representing other than theoretical recovery, it is essential to show that this factor does in fact remain constant during the differing conditions that may be experienced. The conditions inside the Warburg flasks, however, are quite rigidly defined and the only variables are the concentration of amino acid and the concentrations of other amino acids also present by reason of the limitations of the chromatographic separation.

Determinations carried out over a wide range of concentration did not show any variation in percentage carbon dioxide recoveries, except in the case of serine and of threonine (Table 2) where the variations are not large; furthermore, serine and glycine are completely indifferent to each other's presence in their reaction with chloramine-T.

The method was applied to egg albumin and insulin hydrolysates; no difficulties arose except on

account of the very high tyrosine of insulin, which has been mentioned above. There would be nothing to prevent the accurate determination of tyrosine in other materials containing a moderate proportion of this amino acid; this has been confirmed in this laboratory in work on plant extracts.

It is of interest to compare the protein results with those obtained by other workers. For egg albumin, the list of references is by no means exhaustive, but there is generally good agreement with results either from microbiological assay or by chemical methods, although it must be admitted that the range of published figures is often disconcerting. The disparity between the first and second determinations of methionine is probably due to the ease with which this amino acid is oxidized, the diminution being a measure of the extent of oxidation rather than experimental error. Stein & Moore (1948) have stabilized methionine by the inclusion of 2:2'-thiodiethanol (thiodiglycol) in their solvent. Comparison of insulin figures has been with those implied by partial hydrolysis studies (Sanger & Tuppy, 1951; Sanger & Thompson, 1953) (Table 10). Agreement is excellent except for the threonine value.

It is felt that the method described may well be capable of further refinement. The use of longer papers, or the elaboration of a third solvent system to enable tyrosine and methionine to be determined directly might well be advantageous. Nevertheless, the method as described would appear to be relatively simple and of an accuracy great enough to allow its application to many biochemical problems.

SUMMARY

1. Paper partition chromatography has been applied to the separation of the neutral amino acids isolated by ionophoresis. The method has particular value for the free amino acids to be found in plant extracts.

2. Determination of the separated amino acids eluted from the chromatograms depends on the evolution of carbon dioxide in a Warburg respirometer following oxidation with chloramine-T. The following degree of accuracy has been achieved in the analysis of made-up mixtures of amino acids within the optimum range for each amino acid of 0.08–0.25 mg. N: leucine + isoleucine, valine proline, alanine and glycine ($10 \pm 0.3\%$); isoleucine ($99-93\%$); leucine ($106-97\%$); threonine (in absence of hydroxyproline), serine, phenylalanine ($100 \pm 5\%$); tyrosine (in absence of tryptophan) ($100 \pm 8\%$); methionine ($105-90\%$).

3. The method has been applied to hydrolysates of egg albumin and insulin, and the results compared with those obtained by other analytical techniques.

4. It is suggested that, because of its relative simplicity, the method is suitable for application to routine procedures.

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The Carotenoids of the Carapace of the Echinoderm *Ophidiaster ophidianus*

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In 1934 Karrer & Benz isolated astacin from the bright pink shells of *Ophidiaster ophidianus*; it is now known (Kuhn & Sørensen, 1938) that astaxanthin is the naturally occurring pigment and that astacin is a manipulative artifact. Karrer & Benz did not report the presence of any other carotenoid. As the importance of the presence of trace carotenoids in understanding the metabolism of carotenoids is increasing, it was desirable to find out if *Ophidiaster ophidianus* accumulated carotenoids other than astaxanthin.

EXPERIMENTAL

Material. The material was collected in Galli of Positano (Naples), and two groups of fourteen animals were examined. The animals were all about 14 cm. in diameter.

Extraction of material. The animals were opened and the internal organs removed completely and discarded. The clean shells were covered with acetone and allowed to stand for 5 min. at room temperature. The acetone was then removed by decantation and the carapaces were re-extracted with further quantities of acetone (usually 3–4 times) until all the pigments were removed. The combined acetone extracts were concentrated at room temperature at the

pump, diluted with an equal volume of water and the pigment was extracted with diethyl ether. The ether extract was dried over anhydrous Na_2SO_4 and the solvent then removed under N_2 . The residue was redissolved in light petroleum (b.p. 35–50°) and subjected to chromatographic analysis. When it was not possible to examine the extracts immediately, they were stored below –20° in an ice-box.

Chromatographic analysis. Six separate chromatographic analyses were carried out (on portions of the two extracts). The results were identical in each case. The separations were carried out as follows:

(1) A preliminary separation of the pigment mixture was carried out on CaCO_3 (British Drug Houses Ltd., A.R. grade), using light petroleum (b.p. 35–50°) containing 4% (v/v) of ethanol as developer. The resulting chromatogram is described in Table 1.

(2) Fraction 1A was rechromatographed on alumina (Merck-Brockmann) deactivated by suspending it in methanol for about 30 min., filtering off the methanol and drying overnight at room temperature (Goodwin, 1952a). Light petroleum, containing different concentrations of diethyl ether, was used as developer. Eight fractions were obtained as recorded in Table 2. Fractions 2A and 2B were separated using pure light petroleum and passed straight through the column. On development of the column with light petroleum containing 1.5% (v/v) of diethyl ether,