unknown and the marker appeared as one single spot it was assumed that the unknown sugar was identical with the marker. On all chromatograms run with four different solvent mixtures there appeared an unidentified spot which reacted with *p*-anisidine hydrochloride to give a pale yellow colour and a fairly strong bluish white fluorescence in the ultraviolet light. Its R_f value of 0.05–0.11 in all four solvents tested was different from that of a series of known hexoses and pentoses.

It is of interest to find that the sugars, galactose, rhamnose and xylose, which were present in the hydrolysates of *Nitrosomonas* were also found by Hough, Jones & Wadman (1952) in hydrolysates of carbohydrates extracted from the freshwater alga *Nostoc*. These carbohydrates contain in addition glucose, which could not be detected in our hydrolysates, uronic acids, and an unidentified component. From the information available it is not possible to obtain any indication as to whether the unidentified

component in the *Nitrosomonas* and *Nostoc* hydrolysates could be the same.

SUMMARY

1. Hydrolysates of whole cells of *Nitrosomonas* have been investigated for amino-acids and sugars.

2. The results of a quantitative study of the amino-acid composition were compared with figures for *Corynebacterium diphtheriae* and *Chlorella vulgaris*.

3. Four sugars were detected on paper chromatograms. Their chromatographic properties were identical with those of galactose, ribose, rhamnose and xylose. In addition an unidentified spot was found.

The author wishes to thank Prof. W. O. Kermack, F.R.S., and Dr H. Lees for their advice and encouragement throughout this work. He also wishes to acknowledge the receipt of a grant from the Agricultural Research Council.

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A Radiochemical Method for the Micro-estimation of α -Amino-Acids Separated on Paper Partition Chromatograms

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(Received 16 July 1952)

Consden, Gordon & Martin (1944) successfully separated mixtures of amino-acids on paper partition chromatograms and thereby provided a sensitive method for the qualitative analysis of protein hydrolysates. Although the amounts of amino-acids thus separated were only of the order of micrograms, the elegance of the technique stimulated many workers to attempt their quantitative estimation. Bull, Hahn & Baptist (1949) used a standard method for the colouring of amino-acid spots on paper chromatograms with ninhydrin, and estimated their amino-acid contents by scanning

them with a photometer. The differences in the size of amino-acid spots on chromatograms formed the basis of the method used by Fisher, Parsons & Morrison (1948), who showed that the area of a spot was linearly related to the logarithm of its amino-acid content. Between pH 8 and 12 cupric ion will react with α -amino-acids to give complexes of the type $(H_2N.CHR.COO)_2Cu$. This reaction was employed by Martin & Mittelmann (1948) and Woiwod (1949), who used copper phosphate suspensions at pH 9 to form the soluble copper complexes of amino-acids; the former authors estimated

the copper in the complexes polarographically and the latter by a colorimetric method. Both these methods are essentially modifications of that used by Pope & Stevens (1939).

When radioactive isotopes became generally available, they were applied to the microanalysis of amino-acid mixtures. Two such radiochemical methods have already been described. Keston, Udenfriend & Levy (1947, 1950) converted amino-acids into their *p*-iodobenzenesulphonyl derivatives which were labelled with ^{35}S or ^{131}I or with both. The radioactive derivatives were separated on paper chromatograms and the amino-acids estimated by scanning the spots systematically with a Geiger-Müller counter. Wieland, Schmeiser, Fischer & Maier-Leibnitz (1949), after separating amino-acids in the conventional way on chromatograms, allowed a solution of copper acetylacetonate containing ^{64}Cu slowly to ascend the paper, thereby forming radioactive copper-amino-acid complexes *in situ*. The amino-acids were then estimated by scanning the paper with a Geiger-Müller counter.

We now describe a method similar to that of Martin & Mittelmann (1948), with the important modification that the copper phosphate suspension was prepared from cupric chloride containing ^{64}Cu . The results obtained in applying the method to the analysis of salmine sulphate are also described.

EXPERIMENTAL

On beginning this work we envisaged a simple modification of the method of Woiwod (1949) by incorporating ^{64}Cu into the copper phosphate suspension, and estimating the amount of Cu in the soluble copper complexes of amino-acids radiometrically. However, when we prepared the copper phosphate suspension according to Woiwod's directions, we were unable to reproduce the *F* ratios, of weight of α -amino N to weight of Cu reacting, which he obtained for various amino-acids. Hence we experimented with several methods of precipitating copper phosphate, and suspending it in different buffer systems, finally adopting the procedure described below, which in our hands has given the most satisfactory results. Even so, differences in properties of phosphate suspensions were noted. The average values of the *F* ratio for glycine, alanine, valine, serine, histidine and aspartic acid, which we obtained using phosphate suspensions prepared by the same procedure but at different periods, were in accord with those given by Woiwod (1948). However, the deviations from the average value for any particular amino-acid were between 5 and 8%. These variations can only be attributed to variations in the phosphate suspensions, and may be due to differences in grain size. Since the half-life of ^{64}Cu is short (12.8 hr.), it was necessary to prepare fresh suspensions weekly, and we were unable to use them for more than 3 days. Thus we were restricted to a small number of determinations with each suspension. In early work on the determination of amino-acids in spots cut from one-dimensional paper partition chromatograms, we used the average *F* ratios for amino-acids found by Woiwod and by us, but the results were

inconsistent, recoveries being approximately $100 \pm 8\%$. This was to be expected in view of the inconsistency of the *F* ratio previously described. Considering the small amounts of amino-acid estimated ($1\text{--}10\text{ }\mu\text{g.}$ of α -amino N), the accuracy obtained was reasonable, but insufficient for our purposes. The procedure was abandoned in favour of the one outlined below, in which any slight variations between copper phosphate suspensions are eliminated.

METHOD

Preparation of copper phosphate suspension containing ^{64}Cu . 0.5 g. of Cu foil, which had been irradiated at the Atomic Energy Establishment, Harwell, was dissolved in the minimum of 5N- HNO_3 , 10 ml. of 10N-HCl were added and the solution evaporated to dryness. A further 5 ml. of HCl were added, the solution again evaporated, and the dry chloride dissolved in 50 ml. of distilled water. The resultant solution contained about 10 mc. of ^{64}Cu .

10 ml. of the radioactive CuCl_2 solution, and a solution of 1.1 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 15 ml. of distilled water, were run simultaneously from two burettes at controlled rates into 150 ml. of stirred distilled water, so that at no time was there a large excess of cupric ion over phosphate ion. The pH was adjusted to 9 by the addition of 0.1N-NaOH and the copper phosphate precipitate centrifuged off, washed with 2% (w/v) aqueous $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ containing 17.5 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O/l.}$ and suspended in 200 ml. of this solution. The suspension was allowed to 'age' overnight before use.

Chromatographic technique and method of estimation. The amino-acids were separated on one-dimensional chromatograms. A sheet of Whatman no. 4 or no. 3 MM paper ($22 \times 22\text{ in.}$) was divided by thin pencilled lines into eleven serially numbered strips of equal width; measured samples of the solution of amino-acids to be analysed were applied to strips 8, 10 and 11 by means of a capillary micropipette. Progressively increasing amounts of a control mixture containing the same amino-acids in approximately the same proportions as those in the mixture under examination were applied to strips 1, 2, 4, 5 and 7. Known amounts of the same control mixture were applied to strips 3, 6 and 9 in approximately the same amount as on strip 7. In all cases the solutions were applied as circular spots in the centres of the strips, along a line drawn 7 cm. from one edge of the paper. After development the chromatogram was dried and strips 3, 6 and 9 were cut out, sprayed with ninhydrin and heated to reveal the positions of the amino-acid spots. The strips were then re-aligned with the remainder of the chromatogram, and their amino-acid spots used as guides to cutting the spots on the other strips. Care was taken that the paper cuts carrying the spots for any one amino-acid should all have the same area.

The paper cuts were divided into thin strips, dropped into stoppered Pyrex test tubes, and 2 ml. of 0.18M- Na_2HPO_4 solution added to each tube. After standing overnight, 2 ml. of the radioactive copper phosphate suspension were added to each tube, and they were shaken intermittently over a period of 30 min. The mixtures were filtered through pleated Whatman no. 42 papers and a 1 ml. sample removed from each filtrate. These were counted in a polythene dish beneath a Geiger-Müller counter, and the counts corrected for the decay of ^{64}Cu . Standard curves were then plotted for each amino-acid, showing the amount as $\mu\text{g.}$ of α -amino N

against the corresponding counts/min. The amino-acid contents of the mixture under analysis were then read off by reference to these curves.

Radiochemical assay of ^{64}Cu . In preliminary experiments, 2 ml. of the radioactive filtrates containing the copper-amino-acid complexes were diluted to 10 ml. with distilled water and counted in a Twentieth-Century Electronics Type M. 6 liquid counter. We found that there were several drawbacks to this counting method. The window thickness of the counter is of the order of 35 mg./cm.² which appreciably reduces the β -particle counts from ^{64}Cu . Moreover, after every count with an active sample, and subsequent rinsing of the counter with distilled water, the background was invariably slightly higher than before. This 'memory effect' due to adsorption of Cu on the glass walls could be effectively removed by cleaning the counter with HCl, but the time involved in this procedure reduced that available for counting. We next prepared solid samples by evaporating 0.2 ml. portions of the filtrates to dryness in aluminium trays under a radiant heater, and counted them beneath the window of a G.E.C. Type G.M. 4 counter. The trays were 20 mm. in diameter and 2 mm. deep, and were pressed out from 26-gauge aluminium sheet, which is sufficiently thick to give saturation back-scattering for the β -particles from ^{64}Cu (Yaffe & Justus, 1949). Inspection of the samples prepared in this way showed that they were far from uniform in thickness, most of the deposits being concentrated around the tray periphery, but all the counts obtained lay on the calibration curve within $\pm 3\%$, when counted to $\pm 1\%$ statistical counting error. The most satisfactory method of assaying ^{64}Cu in our experience, however, is to pipette 1 ml. of radioactive solution into a polythene dish and count it beneath an end-window counter of the G.E.C. G.M. 4 or E.M.M. 2 types. The polythene dishes we used were turned from 1 in. diameter polythene rod, and had internal diameter 23 mm. and depth 7 mm. We tested this method fairly rigorously by counting twenty 1 ml. samples of a ^{64}Cu solution successively in the same polythene dish; the counts obtained showed no variations other than those to be expected from a Poisson distribution. The polythene dishes retain traces of ^{64}Cu , but this can be removed by a brief wash in an acid CuCl_2 solution followed by a distilled-water rinse. The method is recommended for the routine radio-metric assay of liquid samples.

RESULTS

The results showed a linear relationship between the amounts of amino-acid and the counts given by the ^{64}Cu in the corresponding copper-amino-acid complexes. Fig. 1 shows this relationship plotted for DL-alanine. Similar curves were obtained for glycine, DL-valine, DL-isoleucine, L-leucine, DL-proline, DL-serine and L-arginine, and for L-cysteic, L-aspartic and L-glutamic acids. In general, the amounts of amino-acids used were in the range 0–12 μg . of α -amino N, but no significant deviation from linearity has been found for the range 0–50 μg . of α -amino N.

The estimation of isoleucine, valine and arginine in salmine sulphate

Analyses of isoleucine and valine in a mixture of amino-acids made up to simulate a hydrolysate of salmine sulphate. As a preliminary to the estimation of isoleucine and valine in a hydrolysate of salmine sulphate the percentage

recoveries of these amino-acids from a mixture of the constituent amino-acids of salmine, in the approximate proportions in which they occur in the protamine, were determined. A mixture of the seven amino-acids, DL-isoleucine, DL-valine, DL-alanine, glycine, DL-proline, DL-serine and L-arginine in the molecular ratios of 1:3:15:1:4:6:50 was prepared, and run on one-dimensional paper chromatograms against a mixture of DL-isoleucine and DL-valine in the molecular ratio of 1:3:15 using collidine as developing solvent. Whatman no. 3 MM paper was used in preference to Whatman no. 4, since the

Fig. 1. Curve showing the counts given by the copper complex of increasing amounts of alanine run on a one-dimensional butanol chromatogram.

former gives compact spots with much heavier amino-acid loads than the latter; even so the amounts of isoleucine which could be satisfactorily separated were only of the order of 1.5 μg . of α -amino N. This amount corresponds to a total of 1.2 mg. of amino-acid mixture/spot. The results of eight analyses of isoleucine and valine appear in Table 1. It will be noted that all the isoleucine recoveries with one exception lie within the range $100 \pm 3\%$ and all the valine recoveries within the range $100 \pm 2\%$.

Table 1. *Estimations of DL-isoleucine and DL-valine separated from a mixture of DL-isoleucine, DL-valine, glycine, DL-alanine, DL-proline, DL-serine and L-arginine*

(For details see text.)

Sample no.	$\mu\text{g. N}$			
	Isoleucine		Valine	
	Applied	Found	Applied	Found
1	1.40	1.38	4.48	4.48
2	1.53	1.47	4.90	5.00
3	1.55	1.57	4.96	4.95
4	1.51	1.48	4.83	4.92
5	1.50	1.44	4.80	4.90
6	1.50	1.54	4.80	4.88
7	1.21	1.24	3.89	3.81
8	2.06	2.10	6.60	6.55

Analyses of isoleucine and valine in salmine sulphate hydrolysates. The isoleucine and valine contents of two hydrolysates of salmine sulphate were determined. The first hydrolysate was prepared by refluxing 1.351 g. of dry salmine sulphate in 20 ml. of 5N-HCl for 24 hr.; in the second, 1.425 g. of dry salmine sulphate with 28.1 mg. added DL-isoleucine were treated in the same way. The

salmine sulphate used for these hydrolysates was air-dry and its moisture content was determined by drying a previously weighed portion to constant weight in an oven at 102°. The isoleucine added to the second hydrolysate was sufficient approximately to treble the isoleucine content. Five analyses of each of the amino-acids were made on each hydrolysate, the separations being carried out on Whatman no. 4 one-dimensional paper chromatograms run in collidine. A mixture of isoleucine and valine in the molecular ratio 1:3.15 was used to plot the standard curves. The SO_4^{2-} content of the salmine sulphate used was estimated gravimetrically as BaSO_4 and found to be 19.9%, and its total N content estimated (Kjeldahl) to be 24.2%. By employing a factor of 0.797 to convert the weight of salmine sulphate to salmine, the isoleucine and valine contents of the hydrolysate were determined as g. of amino-acid/100 ml. of salmine, and are given in Table 2.

Table 2. *Analyses of isoleucine and valine in two salmine sulphate hydrolysates*

Sample no.	g./100 ml. salmine (free base)			
	Hydrolysate of salmine sulphate alone		Hydrolysate of salmine sulphate with added isoleucine*	
	Isoleucine	Valine	Isoleucine	Valine
1	1.30	3.70	3.86	3.70
2	1.29	3.58	3.91	3.70
3	1.26	3.57	3.85	3.64
4	1.26	3.72	3.84	3.65
5	1.25	3.60	3.88	3.68

* 2.60 g. isoleucine/100 g. salmine (free base).

From Table 2 the isoleucine content of the normal salmine sulphate hydrolysate is 1.27 ± 0.01 g. and the valine content 3.65 ± 0.03 g./100 g. of salmine, where the errors are the standard errors of the mean. The isoleucine and valine contents of the hydrolysate, to which isoleucine was added before hydrolysis, are 3.87 ± 0.01 g. and 3.70 ± 0.01 g./100 g. of salmine respectively. Since the amount of isoleucine added was equivalent to 2.60 g./100 g. salmine, it is evident that the recovery of isoleucine from the hydrolysate is 100%.

Estimation of arginine in salmine sulphate hydrolysate. The normal salmine sulphate hydrolysate (i.e. with no added isoleucine) used above was diluted 1:10 with water, the resultant solution applied to Whatman no. 4 paper with the necessary number of arginine standard spots; the chromatograms were developed in collidine. The average of ten analyses for arginine was 85.4 ± 0.2 g. of arginine/100 g. of salmine, the highest value determined being 87.8 g. and the lowest 84.6 g./100 g. of salmine respectively.

Losses of amino-acid during paper chromatography

The possible losses that occur during the chromatography of amino-acids were investigated as follows. Known amounts of an amino-acid were run on a one-dimensional chromatogram. To certain of the strips, however, no amino-acid was applied before running, but paper cuts were made in the usual way. Known amounts of amino-acid were now added, converted into the copper complex, and counted. By

reference to a standard curve drawn from these results the percentage 'recovery' of amino-acid which had been run on the chromatogram could be calculated: for collidine it was $100 \pm 3\%$, for phenol $98 \pm 3\%$, for *n*-butanol $100 \pm 3\%$.

There has been considerable discussion as to the extent of the loss of amino-acid that occurs during paper chromatography, the principal sources of loss being attributed to (a) loss during development of the chromatogram (Woiwod, 1949), and (b) loss due to partial decomposition of amino-acid during drying of the chromatogram (Fowden, 1951; Brush, Boutwell, Barton & Heidelberger, 1951; Novellie, 1950). Our results are incompatible with the occurrence of appreciable losses of type (a). It is reasonable to suppose that the magnitude of losses of type (b) will depend not only on the temperature at which drying is carried out, but also on the speed of drying, i.e. the time during which the amino-acid is in contact with hot solvent. This latter factor is greatly dependent on the velocity of the air current in which drying takes place. Our chromatograms were dried in a powerful blast of air which gradually attained a temperature of 100°, and under these conditions the solvent on the paper is effectively removed before high-temperature reaction with the amino-acid spots can occur.

DISCUSSION

The present method for the micro-estimation of amino-acids separated on one-dimensional paper chromatograms compares favourably in accuracy with other methods. In practice we found the accuracy to be $\pm 3\%$ on amounts of 1 $\mu\text{g.}$ of α -amino N and $\pm 2\%$ for amounts of 2 $\mu\text{g.}$ of α -amino N and above. This degree of precision is not increased if amounts up to 50 $\mu\text{g.}$ of α -amino N are used. However, the accuracy attainable is better than has been given by any other method employing paper partition chromatograms to separate amino-acids and the method is more sensitive than most of those hitherto described. Furthermore, the accuracy is comparable with that of other methods of amino-acid analysis, i.e. Stein & Moore (1948) claim recoveries of $100 \pm 3\%$ for the amino-acids which they separate by their starch column chromatographic technique.

The method has the advantage that large numbers of accurate analyses can be carried out in a relatively short time, for, although the half-life of ^{64}Cu is only 12.8 hr. we have been able to use the radioactive copper phosphate suspensions over a period of 2 days, during which about thirty-six analyses were made. The principal limitation of the method in its application to one-dimensional paper chromatograms is the fact that, although spraying with ninhydrin may clearly distinguish between two amino-acid spots, the separation between them may not be sufficient for clean cuts to be made solely by reference to a guide strip.

Isoleucine, valine and arginine are the only amino-acids of the seven present in hydrolysates of salmine sulphate which we have separated sufficiently well

Table 3. *Amounts of isoleucine, valine and arginine present in salmine*

Amino-acid	Amino-acid (g./100 g. of salmine)						
	Tristram (1947)	Hamer & Woodhouse (1949)	Block <i>et al.</i> (1949)	Velick & Udenfriend (1951)			Present authors
				Microbio- logical assay*	Isotope dilution	Ion exchange	
Isoleucine	1.61	1.8	1.2	1.1	N.d.	N.d.	1.27
Valine	3.1	3.8	4.1	4.3	5.0	4.7	3.65
Arginine	85.2	87.2	N.d.	N.d.	N.d.	84.5	85.4

* Microbiological analyses by Dr G. B. Ramasarma.
N.d. = not determined.

on one-dimensional chromatograms for accurate determination. Our estimates of the amounts of these amino-acids present in salmine are shown in Table 3 together with the analyses of Tristram (1947), Hamer & Woodhouse (1949), Block, Bolling, Gershon & Sober (1949) and Velick & Udenfriend (1951). We find that isoleucine, valine and arginine are present in salmine in the ratios of 1:3:50 (the exact isoleucine to valine ratio from our figures being 1:3.22), and such ratios would require a somewhat higher molecular weight for salmine than the figure of 8000 calculated by Tristram (1947); who found the ratios of these amino-acids to be 1:2:40. The salmine sulphate we used was prepared by Boots Pure Drug Co. Ltd. from salmon milt obtained from Vancouver, British Columbia; it seems probable, therefore, that the salmon came from the Fraser River. Tristram (1949) used salmine sulphate prepared from Spring or Chum salmon.

A full analysis of salmine sulphate has been carried out in these laboratories, and the results of

this analysis, together with a fuller discussion of the salmine problem, will form the subject-matter of another paper.

SUMMARY

1. A radiometric method is described for the micro-estimation of amino-acids separated on one-dimensional paper chromatograms. They are converted to their copper complexes with copper phosphate containing ^{64}Cu ; 1 $\mu\text{g.}$ of α -amino N can be estimated with an accuracy of $\pm 3\%$ and 2 $\mu\text{g.}$ of α -amino N and above, with an accuracy of $\pm 2\%$.

2. Analyses of isoleucine, valine and arginine in salmine sulphate are reported.

The authors wish to express their thanks to the Director of Research and Council of the Wool Industries Research Association for permission to publish this paper, to Dr A. J. P. Martin, F.R.S., for criticism and advice and to Mr G. R. Lee, Miss J. Shaw and Miss B. Skinner for help with the experimental work.

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