6. Only relatively small amounts of vitamin A were found in the other tissues examined.

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A Method for the Estimation of Micro Amounts of Amino Nitrogen and its Application to Paper Partition Chromatography

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Since the introduction of paper partition chromatography (Consden, Gordon & Martin, 1944) it has been possible to separate completely many amino-acids and peptides from complex mixtures. This has focused attention on the need for quantitative methods for estimation of micro amounts of these substances after their separation on paper chromatograms. The method of Pope & Stevens (1939) has proved of great use in the estimation of amino nitrogen at macro and semi-micro levels. Preliminary notes of its use in a modified form for determining micro amounts of amino-acids separated by single-dimensional paper chromatography have already been given (Woiwod, 1948*a*, *b*). The present paper gives fuller details of the method, together with some preliminary results.

In the method of Pope & Stevens (1939) amino and 'peptide amino' nitrogen are estimated by allowing a suspension of copper phosphate to react with the material being investigated at pH 9·3, and, after filtration, the copper in the soluble complexes formed is estimated iodometrically. The terms 'amino nitrogen' and 'peptide amino nitrogen' just mentioned are used in this paper in the following sense. The groupings with which the copper phosphate reacts in proteins and their breakdown products are the α-amino (or imino) group of free amino-acids and the free amino groups of peptides and proteins, i.e. any not involved in peptide linkage. The nitrogen of the reacting group in free amino-acids is designated α-amino nitrogen and the nitrogen of the free amino groups in peptides and proteins is designated here as 'peptide' amino nitrogen. Little is known about the reactions of copper with peptide amino nitrogen. The limitation this imposes when interpreting results with partial digests of peptides and proteins is discussed later.

EXPERIMENTAL

The method of Pope & Stevens (1939) can be modified so that micro amounts of amino N can be estimated by using the diethyldithiocarbamate reaction (Callan & Henderson, 1929) to determine the Cu in the soluble copper complexes. The method consists of the following stages: (*a*) the amino-acid or peptide is allowed to react with a suspension of copper phosphate and the excess of the latter is then filtered off; (*b*) the Cu in a measured sample of the filtrate is determined by means of sodium diethyldithiocarbamate; (*c*) the Cu so determined is related to amino or peptide N, or weight of amino-acid or peptide, by means of a standard curve or use of a factor.

The ratio, F, of weight of α-amino N to weight of Cu reacting has been determined for a number of amino-acids by this method (Woiwod, 1948*b*). The copper phosphate suspension of Pope & Stevens (1939) was used in the preliminary work, but its use had several disadvantages at micro levels. These were: (*a*) a considerable deviation of the value of F from the theoretical value of 0·44 for complexes of the type A_2Cu was observed with most amino-acids, and the ratio was not constant from acid to acid; (*b*) the relationship between α-amino N and Cu was not linear; (*c*) the reagent did not keep well and needed to be freshly prepared for each set of determinations; (*d*) the reaction had a large temperature coefficient; and (*e*) the blank value given by the reagent was large.

By omitting the $Na_2B_4O_7$ and adding Na_2HPO_4 to the Cu reagent a linear relationship between amino nitrogen and copper over a considerable concentration range was obtained, and the value of F for many amino-acids was much nearer the theoretical value of 0·44. Thus it was possible to use this

factor for converting copper to amino N in comparative work not requiring the highest accuracy, and with mixtures of amino-acids such as complete hydrolysates of protein. The elimination of $Na_2B_4O_7$ also increased the stability of the reagent, which was then no longer affected by temperature, and blanks were low and consistent. It was, however, not possible to obtain the same value of F for all amino-acids. Mixing the components of the new reagent at room temperature gave a copper phosphate suspension the pH of which slowly increased on standing. At the same time the factor F obtained with standard amino-acid solutions slowly decreased, and with glycine most nearly approached the theoretical value of 0·44 when the reagent had stabilized. This stabilization was assisted by heating; refluxing the reagent for 1 hr. and leaving to 'age' 24 hr. before use gave a satisfactory final product. Such a reagent has been used over a period of 1–2 months with no apparent change in properties when tested with a standard glycine solution. For determining amounts of α-amino N of the order of 1 mg./ml. and determining Cu iodometrically the original reagent of Pope & Stevens (1939) was more satisfactory as it gave a greater uptake of Cu/mol. of amino N than the modified reagent.

METHOD

Apparatus. All glassware must be thoroughly cleaned to remove traces of Cu and a routine of cleaning rigidly followed if blanks are to be minimal and consistent. After use all glassware should be washed first with soap and water, then rinsed and transferred to a Pyrex glass vessel to soak overnight in N-HCl. After soaking, all tubes are tested with an acid solution of sodium diethyldithiocarbamate, rinsed with distilled water (less than 0·02 μg. Cu/ml.) and dried, and funnels are rinsed with distilled water.

Distilled water. All reagents are prepared with good grade distilled water (less than 0·02 μg. Cu/ml.). Where glass-distilled water is necessary its use is mentioned in the text. The following reagents are required:

Cupric chloride. Dissolve 27·3 g. $CuCl_2.2H_2O$ (A.R.) in boiled distilled water and make up to 1 l.

Trisodium phosphate solution. Dissolve 25·6 g. Na_2HPO_4 (A.R.) in 500 ml. boiled distilled water, add 180 ml. N-NaOH and make up to 1 l. with boiled distilled water.

Disodium hydrogen phosphate. Dissolve 25·6 g. Na_2HPO_4 (A.R.) in boiled distilled water and make up to 1 l.

Sodium diethyldithiocarbamate. Dissolve 2·0 g. in 100 ml. distilled water and filter. This solution precipitates slowly on standing and is refiltered immediately before use.

Standard copper solution. Dissolve 3·928 g. $CuSO_4.5H_2O$ (A.R.) in boiled glass-distilled water, add 1 ml. concentrated H_2SO_4 (A.R.) and make up to 1 l. This solution is diluted 1 in 10 with glass-distilled water for use. One ml. of this diluted solution contains 100 μg. Cu.

Copper phosphate suspension. The $Na_3(PO_4)_2$ solution (1 vol.) is added to 1 vol. of the $CuCl_2$ solution and well mixed. The Na_2HPO_4 solution (4 vol.) is then added, mixed, and the mixture boiled under reflux for 1 hr. The resulting copper phosphate suspension is allowed to 'age' for 24 hr. before use.

Preparation of standard copper curve

All water used in the preparation of the standard curve should be glass distilled. Volumes of the standard copper solution containing between 1 and 100 μg./Cu are pipetted into 5 ml. distilled water, then 0·1 ml. sodium diethyldithiocarbamate solution is added and the volume made up to 10 ml. with glass-distilled water. The yellow solution is shaken for 15 sec. with 10 ml. amyl alcohol (A.R.), centrifuged to clear the amyl alcohol layer and the optical density of this layer determined by means of a Spekker absorptiometer using blue glass filters no. 7 (maximal transmission at about 440 mμ.). The curve relating Cu to optical density is linear up to 6·0 μg. Cu/ml.

Preparation of standard amino-acid or peptide curve

For the highest accuracy it is necessary to prepare a standard curve for each specific amino-acid or peptide. These curves are reproducible and once prepared only need checking from time to time with a standard solution. Glass-distilled water is used in the preparation of the standard curve. The standard amino-acid or peptide solution is added by means of a Trevan microsyringe (Trevan, 1925) to 2·5 ml. of the Na_2HPO_4 solution, the added volume being 0·1 ml. or less. Not more than 50 μg. α-amino N or 25 μg. of peptide amino N should be added. After thorough mixing, 2·5 ml. of the copper phosphate reagent are added; the mixture is well shaken, allowed to stand for 30 min. and filtered through pleated papers (Whatman no. 42, 9 cm.) which prevent the copper phosphate suspension 'creeping'. The filtrate (2 ml.) is pipetted into 8 ml. of glass-distilled water in a centrifuge tube, 0·1 ml. of the diethyldithiocarbamate solution is added, mixed and stood for 10 min. to allow the reaction to proceed to completion. The solution is extracted with 10 ml. amyl alcohol with shaking for at least 15 sec., followed by centrifugation to clear the amyl alcohol layer. The optical density of the yellow solution is determined and the values recorded as μg. copper by means of the standard curve. A blank determination is carried out in triplicate at the same time, the procedure being identical except that an equal volume of glass-distilled water is added instead of the solution containing the amino N. The blank value is subtracted and the corrected Cu value obtained is multiplied by the dilution factor. Thus a standard curve is obtained covering the range 1–50 μg. of α-amino N or 1–25 μg. peptide amino N/5 ml.

Estimation of amino nitrogen

The procedure is the same as for the preparation of the standard curve, the unknown solution being added in place of the standard amino-acid solution. If it is not possible to keep the volume added to 0·1 ml., any convenient volume may be used, provided a standard curve is prepared using the same volume. Progressive dilution will lead, however, to a falling off in the copper uptake and a loss of sensitivity.

RESULTS

A preliminary survey of some 25 amino-acids and peptides has shown that alanylglycine, histidine, hydroxyproline, aspartic acid, serine, threonine, valine, leucine, isoleucine, glutamic acid, methionine, asparagine, glycine, tyrosine, arginine, phenylalanine, cystine, cysteine, tryptophan, glutamine, α-alanine, proline, lysine and ornithine all react satisfactorily with the reagent (Woiwod, 1948*a*, *b*). Table 1 gives a list of average F values obtained with

some further amino-acids and peptides including 'pantonine' (α-amino-ββ-dimethyl-γ-hydroxybutyric acid; Ackermann & Kirby, 1948; Holly, Barnes, Koniuszy & Folkers, 1948). It was not possible to examine in detail the behaviour of all these substances, but readings were made at 10 and 30 μg. levels of α-amino nitrogen or 5 and 15 μg. of peptide

Table 1. *Ratio (F) for some amino-acids and peptides of weight of α-amino or peptide amino nitrogen to weight of copper reacting*

Compound	Average F value
αγ-Diaminobutyric acid	0·25
α-Amino-n-butyric acid	0·43
α-Amino-ββ-dimethyl-γ-hydroxybutyric acid	0·46
Glycylglycine	0·22
Diglycylglycine	0·23
Triglycylglycine	0·24
γ-Aminobutyric acid	No reaction
4-Aminobutane-1-carboxylic	No reaction
5-Aminopentane-1-carboxylic	No reaction

amino nitrogen, and an average value for the factor *F* obtained from the slope of the curve drawn through these values. In most instances this curve passed through the origin, indicating a linear relationship between copper and nitrogen at least up to 30 μg. of α-amino nitrogen. At micro levels of amino nitrogen those acids which normally form sparingly soluble copper complexes, such as cystine, methionine, leucine and phenylalanine, all react well, presumably because the solubility product of their copper salts is not exceeded.

A more detailed study has been made of glycine, valine and leucine. These give linear curves up to 30 μg. of α-amino nitrogen, but deviate from linearity between 30 and 50 μg. α-amino nitrogen. Excessive dilution of the sodium phosphate solution is inadvisable as the amount of copper reacting/mole of α-amino nitrogen is affected by the strength of this solution. The effect is shown in Table 2, where

Table 2. *The effect of* Na_2HPO_4 *concentration on the amount of copper reacting with glycine and lysine*

Amino-acid	Na_2HPO_4 (% saturation)	Copper uptake (μg. Cu/2 ml.)	F value
Glycine (10 μg. α-amino N/5 ml.)	8	6·4	0·62
	32	7·9	0·51
	60	9·7	0·41
	100	11·4	0·35
Lysine (10 μg. α-amino N/5 ml.)	8	4·8	0·83
	32	5·7	0·70
	60	7·5	0·53
	100	8·8	0·45

increasing strengths of disodium hydrogen phosphate solution up to saturation give increasing copper uptake with standard solutions of glycine and lysine. Ten μg. amounts of α-amino nitrogen were used in every case, the figures in the third column being the copper value in a 2 ml. sample of the filtrate. The blank copper value also rises slowly with increasing phosphate concentration.

It seems unlikely that any adjustment in the composition of the reagent will give the theoretical value of 0·44 with all acids. The final composition chosen is one which gives this value with glycine. This compound was used for much of the exploratory work. Most of the amino-acids tested give values which lie fairly close to this theoretical value, suggesting the formation of complexes of the type A_2Cu. A number of α-amino-acids, however, behave in exceptional ways. Lysine and ornithine both react less readily than other amino-acids, giving an average *F* value of about 0·5, whereas histidine and αγ-diaminobutyric acid have approximately the same *F* value as peptides (0·22), indicating the formation of a complex of the type *A*Cu. Mono-amino-acids with amino groups at other than the α-position show little or no reaction with copper phosphate. β-Alanine reacts very slightly and γ-aminobutyric, 4-aminobutane- and 5-aminopentane-1-carboxylic acids do not react at all. This failure of amino-acids with the amino group at other than the α-position to form copper salts has enabled Dent (1948) to separate such acids from α-amino-acids by running the copper salts of the mixed acids on two-dimensional paper chromatograms. A mono-amino-acid with the amino group at a position other than α will not be affected by copper phosphate treatment, whereas the α-amino-acids will fail to appear in their usual positions and run to one side of the paper chromatogram. All the peptides so far tested have given values of *F* very near that expected for the formation of a complex *P*Cu.

Application to paper chromatography

The copper phosphate reagent reacts satisfactorily with amino-acids separated by paper chromatography (Woiwod, 1948*a*, *b*) and has been used to determine the ratio of leucine to threonine in a sample of polymyxin A (Jones, 1948). The amino-acid is first located on the chromatogram by inspection in ultraviolet light; this involves no destruction of the acids or peptides. The paper is dried for 3–4 hr. at 80° (Woiwod, 1949), and then examined in the dark under illumination from an ultraviolet lamp. The amino-acids and peptides appear as light blue fluorescent spots on a dark purple fluorescent background.

A square of filter paper (approx. $1\frac{1}{2} \times 1\frac{1}{2}$ in.) with the amino-acid or peptide spot on it is cut into strips about $\frac{3}{4} \times \frac{1}{4}$ in. These are dropped as a bundle into a dry test tube $6 \times \frac{5}{8}$ in., 2·5 ml. of Na_2HPO_4 solution is added and the tube left to stand 30 min. with occasional swirling. Copper phosphate suspension (2·5 ml.) is then added and the mixture, after standing a further 30 min., is filtered. The filtrate is treated as described for the determination of amino N. A blank square of paper of the same size is cut from the paper and carried through an identical series of operations. The Cu value obtained after correcting for the blank is converted

to N or weight of material by means of a standard curve. The blank consists mainly of soluble copper from the copper phosphate suspension and is normally about 1·0 μg. copper/ml. of filtrate. A square of Whatman (nos. 1 or 4) paper 1½ × 1½ in. gives no readable copper colour when tested without the addition of copper phosphate.

Early work with glycine and valine using *n*-butanol with ammonia as the solvent give recoveries of amino N between 90 and 95% after chromatography (Woiwod, 1948*a*). After further development of the copper reagent, recoveries from chromatograms were re-investigated. The solvent used was *n*-butanol-acetic acid (Partridge, 1948) run on Whatman no. 4 paper, this combination of solvent and paper being used for routine single-dimensional chromatography in this laboratory. Glycine, valine and leucine were studied. Ten spots of each substance were run for 16 hr., the spots located by ultraviolet light and the amino N determined therein. The recoveries, based on the copper in a 2 ml. sample of filtrate, are shown in Table 3, together with the standard deviation from the mean of the ten results at each level. The results indicated that the percentage recovery decreased with increasing R_F values. 'Collidine' gave a similar result with five spots each of glycine and leucine.

If the loss of amino N increases with the distance the spots travel, then decreasing recoveries should be obtained when the same substance is run on the chromatogram for increasing times. This is the case with glycine (Fig. 1). Samples containing 20 μg. α-amino N were run for various times on Whatman no. 4 paper with *n*-butanol-acetic acid as solvent, the paper removed, dried and determinations of amino N made. There was a progressively poorer recovery with increase in the distance the amino-acid had travelled from the starting line. Little work has yet been done with recoveries from two-dimensional paper chromatograms. It was thought, however, worth seeing whether the method could be applied to such paper chromatograms. A mixture of glycine, valine and leucine was run in duplicate first with *n*-butanol-acetic acid and then with phenol as solvents, in that order. After 16 hr. in each direction on Whatman no. 4 paper, the spots were located by ultraviolet light and the amino N determined. The recoveries are shown in Table 3. Considerably greater losses occurred with the two-dimensional paper chromatograms than in the single-dimensional runs. The losses, again, were greatest with the faster running compounds. It was thought that adsorption was the most likely cause of the losses. It was noted, however, that with single-dimensional paper chromatograms a 'tramline' of fluorescence often extended right to the solvent front. It may therefore be that failure of the acids to reach equilibrium with the solvent in the moving phase is partly responsible for the losses.

The rather large losses of amino N on paper chromatograms, particularly with faster running amino-acids, probably accounts for the larger quantity of amino N needed to obtain good two-dimensional paper chromatograms. The amino-acids, besides being depleted during the run, are also dispersed over three to four times as great an area as compared with a single-dimensional paper chromatogram. For this reason more satisfactory two-dimensional paper chromatograms are obtained when the starting spot is kept as small as possible, preferably 0·1 in. in diameter, or less (Woiwod, 1949).

Table 3. *Recovery of amino nitrogen from chromatograms of single amino-acids on Whatman no. 4 paper*

Solvent	Amino-acid	Approx. R_F value	Before running (μg. Cu/2 ml.)	Recovery (μg. Cu/2 ml.)	Standard deviation (μg. Cu/2 ml.)	Recovery (%)
Single-dimensional paper chromatograms run 16 hr.						
n-Butanol-acetic acid	Glycine	0·2	18·6	17·7	0·3	95·1
	Valine	0·4	28·8	26·1	0·6	90·7
	Leucine	0·6	21·6	17·6	0·8	81·5
'Collidine'	Glycine	0·1	18·6	17·5	—	94·1
	Leucine	0·4	21·6	17·6	—	81·5
Two-dimensional paper chromatograms run 16 hr. each way						
n-Butanol-acetic acid and phenol	Glycine	—	18·6	16·1	—	86·5
	Valine	—	28·8	20·6	—	71·6
	Leucine	—	21·6	13·8	—	63·9

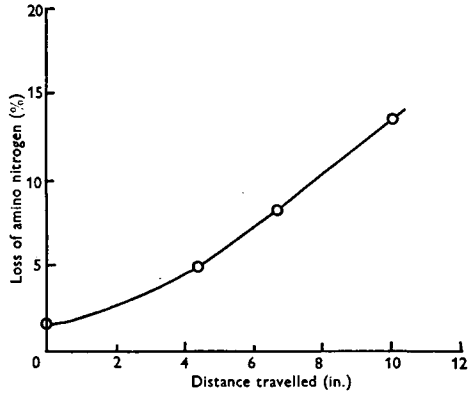

Fig. 1. Curve illustrating the progressive loss of amino N with increasing distance travelled from starting line. Single-dimensional paper chromatogram run with *n*-butanol-acetic acid on no. 4 Whatman paper. Original glycine concentration equivalent to 20 μg. α-amino N.

The destruction of amino-acids by ninhydrin on paper chromatograms

The usual procedure for locating amino-acids on paper chromatograms is to spray with a solution of ninhydrin and then to heat. Coloured spots are obtained which indicate the position of the various amino-acids and peptides. An investigation, by means

of the copper reagent, of the breakdown of amino-acids occurring during ninhydrin colour development, indicated that considerable quantities of amino nitrogen remained after colour development was apparently complete. Before the copper reagent could be applied to ninhydrin-sprayed papers, however, a method had to be found for blocking the reaction between excess ninhydrin on the paper and copper phosphate. The addition of 4·0 g./l. of sodium borate to the disodium hydrogen phosphate solution completely inhibited the reaction. That there was not complete destruction of amino-acids by ninhydrin was noted by Work (1948) who rechromatographed a spot already fully developed by ninhydrin and was able to obtain a coloured spot in a new position after respraying with more ninhydrin and heating. We have confirmed this observation. With glycine it has been found that after spraying with ninhydrin, heating for increasing times and estimating the residual glycine with copper phosphate reagent, there is a rapid initial destruction of amino nitrogen and then breakdown practically ceases. Respraying with ninhydrin solution and reheating causes little further destruction, nor is extra colour development obtained. This behaviour, illustrated in Fig. 2, would

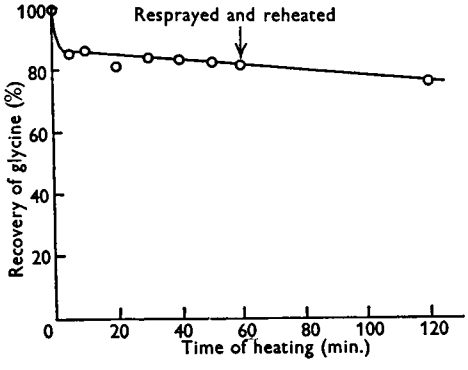

Fig. 2. Residual glycine, by copper phosphate estimation, on no. 4 Whatman paper after spraying with 0·1 % ninhydrin in chloroform and heating. Original glycine concentration equivalent to 20 μg. α-amino nitrogen.

be consistent with the formation of a substance inhibiting the reaction between ninhydrin and glycine on paper. The ninhydrin reaction is undoubtedly complex, as solutions of the coloured spots give a number of coloured bands on an alumina column, and it seems probable that among the products of the reaction such an inhibitor could be present. It was suggested (Woiwod, 1948*a*) that the failure of ninhydrin to destroy more than a portion of the amino-acid in a spot on paper might enable acids to be located by this reagent before quantitative estimation. The difficulty in assessing the degree of breakdown, however, led to abandonment of the method in favour of using ultraviolet light for locating the spots.

DISCUSSION

The copper phosphate reagent described here can be used to determine both the α-amino group of amino-acids, and the free amino groups in polypeptides and proteins which have been called 'peptide amino' groups in this paper. Although two conversion factors are therefore necessary, this raises no problems when determinations are made on solutions of isolated amino-acids or peptides, as the necessary conversion can in most cases be achieved by means of standard curves prepared beforehand. With mixtures of α-amino-acids and peptides, however, such as those in partial hydrolysates of proteins, it is not possible to say with certainty what factor should be used for converting copper into amino nitrogen.

Pope & Stevens (1939) followed the digestion of fibrin by trypsin and showed that the results they obtained with their copper reagent agreed with those given by the gasometric Van Slyke method. A conversion factor which assumed that 1 mole of copper was equivalent to 2 moles of amino nitrogen was used. It may be that very long peptide chains reacted under their conditions in such a way that this factor was a fair approximation. However, the few peptides tested at micro levels (Table 1) react as if a complex *P*Cu was formed. By the ninhydrin method of Van Slyke, MacFadyen & Hamilton (1941) it is possible to determine and to correct for free α-amino nitrogen in a complex protein digest, and hence to obtain the peptide amino nitrogen, assuming a factor of 0·22 to hold in all circumstances when converting copper into peptide amino nitrogen. This assumption may not be justified in view of the lack of knowledge existing about the reaction of copper with peptide amino nitrogen. Though the ϵ-amino group of lysine appears to be inactive in the free acid no information is available as to its reaction when the α-amino group is in peptide linkage. Similarly, it is by no means certain how the iminazole group of histidine will react when its α-amino group is in peptide linkage. In practice, however, these limitations are not found to detract from the usefulness of the copper reagent in following the rate of hydrolysis of a protein, when an arbitrary factor of 0·44 for converting copper to amino nitrogen is used. The presence of such acids as histidine or $\alpha\gamma$-diaminobutyric acid in large amounts in peptides or proteins makes it difficult to assess the degree of breakdown of such materials, as both these amino-acids form complexes of the type *A*Cu, and amino-nitrogen figures in excess of the total nitrogen figures can be obtained if the factor 0·44 be used. By plotting amino nitrogen against

time a guide to the completeness of the digestion can be obtained although the figures have no absolute significance. Attention is drawn to these points as ignorance of these effects might lead to faulty conclusions being drawn from experimental results.

The failure to obtain a theoretical conversion factor with many amino-acids is at first sight puzzling, since it appears in some instances as if more copper were incorporated than the formation of the complex A_2Cu would allow. Borsook & Thimann (1932), however, showed that a number of copper complexes of both glycine and alanine can be obtained and can co-exist in solution; similar behaviour with other acids could account for the anomalous results obtained in the present work. Furthermore, it is not certain that the soluble copper salts estimated by the present method correspond to the isolated copper salts of the amino-acids.

SUMMARY

1. A method is described for determining micro amounts of α-amino nitrogen (1–50 μg.) and peptide amino nitrogen (1–25 μg.).

2. The method has been applied to single- and two-dimensional paper chromatograms of a number of α-amino-acids and an evaluation made of the losses involved during chromatography.

3. The destruction of amino-acids on heating the spots on paper chromatograms developed with ninhydrin has also been studied quantitatively. It is suggested that a decomposition product makes the reaction self limiting.

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Long-chain Unsaturated Fatty Acids as Essential Bacterial Growth Factors

SUBSTANCES ABLE TO REPLACE OLEIC ACID FOR THE GROWTH OF *CORYNEBACTERIUM* 'Q' WITH A NOTE ON A POSSIBLE METHOD FOR THEIR MICROBIOLOGICAL ASSAY

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Forty years ago, Fleming (1909) observed the stimulatory effect of oleic acid on the growth of *Corynebacterium acnes*, but it is only recently that the essential nature of long-chain unsaturated fatty acids for the growth of certain organisms has been firmly established. It is now generally accepted that linoleic acid is essential for the proper nutrition of rats (see Hansen & Burr, 1946), while Fraenkel & Blewett (1947) have shown that it (or linolenic acid) is necessary for normal development of the moth *Ephestia kuehniella*. Benham (1941) has reported that oleic acid is essential for the growth of the fungus, *Pityrosporum ovale*, while, amongst bacteria, oleic acid has been found necessary for the growth of some strains of *Corynebacterium diphtheriae* from a small inoculum (Cohen, Snyder & Mueller, 1941), for *Clostridium tetani* (Feeney, Mueller & Miller, 1943), for *Cl. sporogenes* in the absence of biotin (Shull, Thoma & Peterson, 1949), and for the unidentified *Micrococcus* 'C' (Dubos, 1947). There have also been reports, of which the most accurate and complete are those of Williams & Fieger (1946)