The Quantitative Recovery and Colorimetric Estimation of Amino-acids Separated by Paper Chromatography

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Numerous methods have been published for the quantitative estimation of amino-acids after separation by the filter-paper chromatography technique of Consden, Gordon & Martin (1944). In all methods the solvent used in the development of the chromatogram is first removed by drying the paper in a current of hot air at a temperature of 70–100° before the estimation of the amino-acid. Many different types of assay have been used, but they can be divided into three groups. In the first group, the intensities of the colours of the spots produced on the chromatograms following treatment with ninhydrin are compared visually (Polson, 1948) or by photometry (Bull, Hahn & Baptist, 1949). For one estimation a large number of chromatograms are required; an accuracy of 10–15 % has been claimed by different workers. The second group includes assays based on the formation of water-soluble amino-acid copper complexes (2 molecules of α-amino-acid to 1 atom copper). The copper complexes have been estimated by polarography (Martin & Mittelmann, 1948) and by determination of the copper colorimetrically (Woiwod, 1949). The latter has obtained results which appear to be reproducible with an accuracy of 5–10 %. The third group includes methods in which the amino-acids are determined colorimetrically after removal from the chromatograms using a ninhydrin reagent.

The ninhydrin method of Moore & Stein (1948) for estimating amino-acids separated by starch column chromatography is very sensitive, accurate and reproducible. In the present work a slightly modified Moore & Stein reagent has been applied to the estimation of amino-acids separated on filter paper. Preliminary experiments showed that this method was more sensitive than the copper complex method of Woiwod (1949), which may be further complicated by the presence in filter paper of a substance which inhibits the completeness of complex formation between copper and some amino-acids (Fowden, 1949). The ninhydrin method of Naftalin (1948) lacked reproducibility, whilst the method of Awapara (1949), in which 1 ml. of a 10 % solution of pyridine and 2 ml. of a 1 % aqueous solution of ninhydrin were added directly to pieces of filter paper containing the amino-acid cut from the chromatograms, was fairly reproducible and sensitive, but was impaired by high and variable 'paper blanks' due to the presence of absorbed ammonia on the paper.

The Moore & Stein reagent gives approximately twice the colour per mol. of amino-acid obtained using the method of Awapara (1949). The amino-acids were estimated whilst still absorbed on pieces of filter paper cut from the chromatograms, thereby eliminating the capillary method of washing the amino-acids from the paper described by Consden, Gordon & Martin (1947), and considerably reducing the manipulation required. After the development of chromatograms in the phenol-ammonia solvent, the concentration of ammonia absorbed on the surface of the filter paper was considerably increased, giving rise to high and variable paper blanks in the colorimetric estimation. The dilute alkali treatment described later to remove this absorbed ammonia, reduced the paper blanks to a low and constant level for equal sized pieces of paper taken from the same sheet. In the determination of 2 or more μg. α-NH_2-N of an amino-acid, the maximum error introduced into the final estimate by the paper blank was then 3 %.

Various procedures were used to remove the solvents from the developed chromatograms, and are described in the Results section. Only the method found to give quantitative recoveries of amino-acids from the chromatograms is given below in the Methods section.

Artificial mixtures of amino-acids were used in the work reported here, but satisfactory results are being obtained with biological materials after desalting by the procedure of Consden *et al.* (1947).

METHODS

Ninhydrin was most conveniently prepared in large batches by the method of Teeters & Shriner (1933), all traces of SeO_2 being removed by additions of small amounts of $SnCl_2$ and repeated recrystallizations from water before use. Hydrindantin was prepared from ninhydrin by treatment with H_2S (Ruhemann, 1911), being recrystallized from acetone. The Moore & Stein ninhydrin reagent was modified to be saturated with hydrindantin, the reduced form of ninhydrin. This change was found to overcome slight interfering effects produced by small amounts of oxidizing agents present in the paper. The results were then found to be more consistent and maximum colour response per mol. of amino-acid was obtained.

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Ninhydrin reagent. Ninhydrin (4 g.) and hydrindantin (80 mg.) were dissolved in 100 ml. of redistilled methyl cellosolve, shown to be free from oxidizing agents by the starch-iodide test. $SnCl_2.2H_2O$ (160 mg.) was then dissolved in 100 ml. of pH 5 citrate buffer, and this solution was added to the first with stirring. After standing for a few hours the precipitated, excess hydrindantin was removed by centrifugation, and the reagent stored under N_2 until required to ensure that its high sensitivity was preserved. All the chemicals used in this reagent were as free from contamination with NH_3 as was reasonably possible, otherwise high reagent blanks resulted in the colorimetric measurements.

Chromatographic technique. The method was essentially that described by Consden *et al.* (1944). Whatman no. 4 filter-paper sheets (56 × 46 cm.) were used in all experiments. The size of the original spots formed in adding the amino-acids to the chromatograms were kept as small as possible, about 6–8 mm. in diameter. The solvents generally used have been phenol saturated with water at the working temperature (a little NH_3 was added to the tank when using this solvent) and the *n*-butanol-acetic acid mixture described by Partridge (1948). All chromatograms were run for about 18 hr. at 25° in a temperature-controlled room. In the development of some of the two-dimensional chromatograms described later, the running time in the *n*-butanol-acetic acid was increased to give a better separation of the amino-acids.

The phenol solvent was removed from the developed chromatograms as follows. The papers were first allowed to dry in cold air for about 1 hr., when the aqueous portion of the solvent evaporated, leaving the papers dry to the touch. The phenol was then removed by washing the papers twice in a bath of redistilled ether, and the chromatograms were finally dried off in cold air. The *n*-butanol-acetic acid solvent was satisfactorily removed by hanging the chromatograms in a current of cold air for 3–4 hr.

In all cases the chromatograms were then heated at 100° for 15 min. The separated amino-acids could then be located as light-blue fluorescent spots on a dark background when the chromatograms were viewed in ultraviolet light. The amino-acids do not fluoresce if this short heating is omitted. Though Gal (1950) has reported losses of amino-acids of up to 7% during prolonged heating in the presence of filter paper, no measurable losses occurred in this 15 min. heating period.

The areas of the chromatogram shown to contain the amino-acids were then removed, together with other pieces of paper of equal size, which served as paper blanks in the colorimetric determinations.

Removal of ammonia. The pieces of paper taken from the dried chromatograms were folded to a convenient size and placed into separate clean 150 × 16 mm. tubes. 0·1 N-NaOH (0·5 ml.) was added to each tube (this volume was just sufficient to wet the paper uniformly) and the tubes placed in a desiccator *in vacuo* overnight over conc. H_2SO_4 (A.R.). Citric acid was then added to bring the papers to approximately pH 5. Alternatively, a calculated extra amount of citric acid was added to the ninhydrin reagent so that pH 5 was obtained after the addition of the reagent to each tube.

If only a few amino-acid determinations were required at any one time, it was found convenient to remove the absorbed NH_3 by the following method, which eliminated the overnight delay but increased the time taken by each determination. After the addition of 0·5 ml. of 0·1 N-NaOH to each tube, they were placed in a boiling-water bath, and a stream of H_2SO_4-washed N_2 was blown through each tube in turn—2 min. was sufficient to remove all the NH_3.

Colour development. Ninhydrin reagent (1·5 ml.) was added to each tube and they were shaken mechanically for 5–10 min. to extract the amino-acids from the paper. The maximum colour production was not obtained on some occasions when the shaking was omitted. The tubes were then placed in a boiling-water bath for 25 min., when colour production was complete. After cooling, 10 ml. of distilled water were added, the tubes shaken and the contents poured into volumetric flasks. Acetone (10 ml.) was added to each tube to complete the extraction of the colour from the papers. The acetone was poured into the appropriate flask and the contents made up to volume, usually 25 ml., with distilled water. In the estimation of small amounts of amino-acid, 1 ml. of reagent was found to be sufficient, and 10 ml. final volumes were used, the volumes of water and acetone used in the colour extraction being correspondingly reduced. The bluish purple colours produced were estimated using a Unicam Spectrophotometer, the percentage light transmission through a 10 mm. tube of solution at $\lambda = 570$ mμ. being measured, and the corresponding optical densities calculated. These optical densities must be corrected for the optical density due to the reagent and paper blanks determined simultaneously on a blank piece of paper of equal area taken from the same chromatogram.

A standard line relating the calculated optical densities with the corresponding known amounts of α-NH_2-N taken can be constructed for each amino-acid, and unknown amounts of amino-acid determined by reference to this standard line.

RESULTS

The lower limit at which the method can be accurately used was found to be dependent upon the certainty with which the spots could be located after development and drying. Under the conditions described, the relative intensities of fluorescence given by 1 μg. α-NH_2-N of each amino-acid on a one-dimensional chromatogram were compared, and the results are presented in Table 1. The table shows that for many amino-acids this amount approached the lower limit needed for accurate location, and in general larger amounts of amino-acids were necessary when two-dimensional chromatograms were used.

When amounts of amino-acids less than 1 μg. α-NH_2-N were used, a parallel location strip was necessary to determine the final positions of the amino-acids. This location strip was treated with ninhydrin and used as a guide in cutting out the amino-acids from the experimental chromatogram. This method of location reduced the lower limit of possible detection by about a factor of 10, but the line of cutting was subject to an appreciable error, since the positions of the amino-acids were only inferred by reference to another chromatogram.

The colours produced from equivalent amounts of the different amino-acids expressed as a percentage of the colour given by leucine are also given in Table 1. The colour response given by all amino-acids was shown to be unaffected by the presence of paper during the colour development stage.

Table 1. *Relative colour responses and intensities of fluorescence given by equimolar amounts of the amino-acids*

Amino-acid	Colour per mol. of amino-acid. (Leucine taken as 100)	Intensity of fluorescence produced from 1 μg. α-NH_2-N of amino-acid
Aspartic acid	96	+
Glutamic acid	108	+
Cystine (0·5 mol.)	49	?
Serine	99	+ +
Glycine	99	+ +
Threonine	93	+ +
Alanine	102	+ + +
Lysine	114	+ + +
Histidine	106	+ +
Arginine	97	+ + +
Methionine	101	+ + +
Leucine	100	+ + +
Isoleucine	102	+ + +
Phenylalanine	90	+ +
Tyrosine	94	+
Tryptophan	86	?
Valine	106	+ + +
Glutamine	102	+
Proline	32*	−
Hydroxyproline	17*	−

* Colours read at $\lambda = 440$ mμ. and compared with leucine read at $\lambda = 570$ mμ.

+ + +, Strong; + +, moderate; +, weak; ?, doubtful; −, absent.

A convenient working range when using 25 ml. final volumes and 10 mm. tubes was found to be from 1 to 10 μg. α-NH_2-N of an amino-acid. The plot of the calculated optical densities against the corresponding concentrations of the amino-acids were linear over this range. Fig. 1 illustrates this

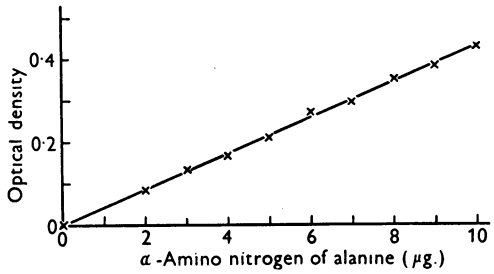

Fig. 1. Optical densities read at $\lambda = 570$ mμ. for increasing amounts of alanine.

point using alanine. With the solutions suitably diluted for use with the spectrophotometer, the linearity of the plot could be extended to 30 μg. α-NH_2-N; this was often an upper limit to the amount of an amino-acid that could be separated from a mixture with good resolution on a paper chromatogram.

The recoveries of standard amounts of amino-acids after flowing on chromatograms were investigated using the standard method of removing the developing solvent by heat. A mixture of aspartic acid, glutamic acid, glycine, threonine, alanine, histidine and phenylalanine, was applied to paper strips (50 μl. spots containing 8 μg. α-NH_2-N of each amino-acid) and chromatographed in phenol. After drying in hot air at 80° for 1 hr. the spots of the separated amino-acids were located using ultraviolet light, and cut out from the papers, together with suitable paper blanks. The recoveries of the amino-acids in all the experiments were based on a comparison of the optical density given by the recovered amino-acid to that given by the standard amount of the same amino-acid when added to a paper blank. The mean recovery values are given in Table 2. In general, the individual results were within 5 % of the mean value.

Table 2 also shows the mean recovery values of the same amino-acids after two-dimensional chromatography. These recoveries were found to be lower than those obtained in one-dimensional chromatography; the scatter of individual results about the mean value was again not greater than 5 %.

Table 2. *Recoveries of some amino-acids taken from developed chromatograms dried at* 80° *for* 1 *hr.*

Amino-acid	Mean percentage recovery after one-way phenol chromatography (four results)	Mean percentage recovery after two-dimensional chromatography (four results)
Aspartic acid	87	80
Glutamic acid	88	72
Glycine	86	76
Alanine	85	71
Threonine	86	73
Phenylalanine	80	60
Histidine	70	49

Using a typical amino-acid, alanine, the percentage recovery was shown to be independent of the amount of amino-acid used, and the distance flowed by the amino-acid on one-dimensional chromatograms. Tables 3 and 4 give the results of these experiments.

The remaining experiments were designed to investigate the nature of the losses encountered during chromatography and to provide a satisfactory method for their elimination, thereby reducing the labour needed for each determination. The apparent loss of alanine during the chromatography process averaged about 8 % when *n*-butanol-acetic acid was the solvent and about 20 % when phenol was used. The constancy of the recovery values obtained for each solvent suggests that the loss could not have been due to trailing of the amino-acid down the paper, for this would have entailed greater losses as the distance of the amino-acid flow increased. Decomposition of amino-acid in the presence of solvent

during the development of the chromatogram was equally unlikely since the amount of decomposition would have been expected to increase, giving a decreased recovery when longer times of running were used.

Table 3. *Recoveries of different concentrations of alanine taken from chromatograms dried at* 90–100° *for* 1 *hr.*

Alanine chromatographed (μg.)	Mean percentage recovery of alanine using *n*-butanol-acetic acid as solvent (four results)	Mean percentage recovery of alanine using phenol as solvent (four results)
10	78	78
20	92	78
40	91	82
50	90	86
60	92	78
70	92	87

Table 4. *Recovery of alanine after flowing for different distances on chromatograms finally dried at* 90–100° *for* 1 *hr.*

Distance travelled by alanine spot (cm.)	Mean percentage recovery of alanine using *n*-butanol-acetic acid as solvent (four results)	Mean percentage recovery of alanine using phenol as solvent (four results)
3	95	.
6	95	75
10	95	81
16	95	77
22	91	.
28	94	77

Losses of amino-acid due to the formation of complexes, having different R_F values, with copper present as an impurity in the filter paper were shown not to occur by comparing the mean recovery of 8 μg. α-NH_2-N of alanine run in phenol to that of alanine run in phenol to which 0·1 % α-benzoin oxime (cupron) was added. The presence of cupron in the solvent would greatly reduce any complex formation between the alanine and copper. The mean values of the percentage recoveries of alanine found for the two solvents were not significantly different ($P > 0{\cdot}05$ for 't' test on difference of means of 76·2 and 79·2 % respectively).

In all the experiments described previously, the paper chromatograms were dried in a hot-air oven at temperatures from 80 to 100° for about an hour. Evidence had accumulated suggesting that the losses were greater when either the drying temperature or time was increased. Using amounts of alanine containing 8 μg. α-NH_2-N the recovery from a chromatogram run in phenol and dried at 80° was shown to fall from 82 % after drying for 1 hr. to 73 % after drying for 3 hr.; again 90 % of the alanine was recovered from a phenol chromatogram dried at a temperature of below 50° for 6 hr. whilst only 69 % was recovered after drying at 100° for 1 hr. Standard amounts of alanine were added to 'blank' areas of the paper chromatograms whilst still saturated with solvent after developing in phenol, and after drying, losses of the added alanine were identical with those found for the alanine which had been run on the chromatogram. When alanine was added to the untreated dry Whatman no. 4 paper, and then subjected to the heat treatment used to dry the chromatogram, no loss occurred.

The losses of amino-acid occurring during chromatography appeared to be due to the partial decomposition of the amino-acids in the presence of solvents at the high drying temperatures used, giving compounds no longer reactive towards ninhydrin, or whose colour response per mol. was much less than that given by the original amino-acids. Drying for longer periods at temperatures below 50° reduced the losses of amino-acid to about 10 %, and for many types of experiment this procedure could be used to obtain rough values in amino-acid assays.

In an attempt to overcome such heat losses Fowden & Penney (1950) suggested the use of ether washing to remove phenol from developed chromatograms, and showed that alanine and phenylalanine could be quantitatively recovered from one- and two-dimensional chromatograms after this treatment. The scope of this earlier work has now been widened to include most of the naturally occurring α-amino-acids.

The recovery values of the amino-acids after flowing on one-dimensional phenol chromatograms are given in Table 5. The recoveries obtained when ether washing was used to dry the papers are compared with those obtained after drying at 80° for 3 hr. Strict comparison was made possible by taking a separate sheet of paper for each amino-acid, and treating in the following manner. Six spots, each containing 8 μg. α-NH_2-N of the amino-acid under investigation, were applied to the top edge of the paper. After developing, each sheet was cut along the direction of flow whilst still saturated with solvent, giving two halves each containing three spots. One half of the paper was then ether-washed and the other heat-dried.

The recoveries of lysine and histidine tended to vary; this was attributed to the tailing that these two amino-acids show in phenol solvent systems. The losses occurring in the hot-air drying of chromatograms varied considerably for the different amino-acids, but as seen from Table 5 most of the amino-acids could be quantitatively recovered from one-way phenol chromatograms provided they were dried by ether washing. The losses of amino-acid produced in the hot-air drying of chromatograms run in *n*-butanol-acetic acid were found to be of the order of 10 %; when these chromatograms were

Table 5. *Comparison of the recoveries of the amino-acids* (8 μg. α-NH_2-N) *from phenol run chromatograms after drying at* 80° *for* 3 *hr. and at laboratory temperature by ether washing*

Amino-acid	Mean percentage recovery using ether washing (three results)	Standard deviation from the mean value (three results)	Mean percentage recovery using hot-air drying (three results)	Standard deviation from the mean value (three results)
Alanine	99	3·7	74	0·9
Phenylalanine	101	0·8	71	2·9
Leucine	97	0·8	66	3·9
Isoleucine	98	1·1	61	2·5
Glycine	98	4·2	70	3·8
Glutamic acid	100	2·5	74	3·4
Aspartic acid	98	1·5	82	3·2
Serine	96	1·8	77	4·8
Threonine	95	3·5	76	1·7
Arginine	98	0·9	73	2·3
Lysine	Variable 85–100	.	About 65	.
Histidine	About 80	.	About 60	.
Glutamine	About 70	.	About 48	.
Methionine	91	2·1	87	1·3
Valine	101	2·6	72	1·4
Tryptophan	89*	1·9	67	3·1
Cysteic acid	86*	2·4	76	4·1

* These amino-acids were located using 0·02% ninhydrin solution, before estimation.

dried in a stream of cold air no losses were encountered.

Table 6 gives the results of similar experiments using 2 μg. α-NH_2-N samples of several amino-acids. There is still quantitative recovery at this lower concentration. The standard deviations from the means (four results) rarely exceeded 5%.

Table 6. *Recovery values of amino-acids* (2 μg. α-NH_2-N) *taken from phenol chromatograms dried by ether washing*

Amino-acid	Mean percentage recovery after drying by ether washing (four results)	Standard deviation from the mean value (four results)
Alanine	95	2·1
Leucine	97	5·9
Glutamic acid	94	3·8
Aspartic acid	92	3·2
Serine	95	4·1
Arginine	100	4·5
Valine	102	1·0

An attempt was made to extend the range of estimation down to 0·5 μg. α-NH_2-N amounts of amino-acid, but at these concentrations the positions of the spots after chromatography had to be found using a location strip, and the recovery values obtained were far more variable. The mean value obtained was therefore subject to an appreciable error.

The ether-washing method was used to dry two-dimensional chromatograms after the separation of ten amino-acids contained in a synthetic mixture; the recovery values obtained for each amino-acid are given in Table 7. The recoveries given are the mean of eight separate experiments.

Table 7. *Recoveries of amino-acids* (8 μg. α-NH_2-N) *from two-dimensional chromatograms dried at laboratory temperature by ether washing*

Amino-acid	Mean percentage recovery after drying by ether washing (eight results)	Standard deviation from the mean value (eight results)
Arginine	100	5·6
Leucine	96	3·8
Valine	96	3·4
Phenylalanine	93	4·5
Alanine	99	2·0
Threonine	100	2·4
Glycine	102	7·6
Serine	102	6·6
Glutamic acid	92	2·2
Aspartic acid	95	3·8

The recoveries of the amino-acids obtained in the experiments described show that any losses of amino-acid occurring during the development of the chromatograms must be extremely small, and for practical purposes may safely be ignored.

When the improvements described for the drying and colour development stages and in the removal of absorbed ammonia are carefully followed, the result of a single assay of an amino-acid in a mixture may be expected to have an error of not more than 5% of the true value.

DISCUSSION

The results obtained in this investigation indicate that the nature of the losses previously encountered in the paper-chromatographic separation of amino-acids may depend largely upon the final method of

estimation used. Results obtained, using the ninhydrin method described here, failed to show any increased loss after longer times of running as found by Woiwod (1949) using a copper complex method of estimation. It is inconceivable that this fundamental difference is due to slight differences in the conditions under which the chromatograms were developed, and a possible explanation for these progressive losses when using the copper method of assay is the presence in paper of a substance which inhibits complex formation (Fowden, 1949). On the other hand, hot-air drying seems to produce much smaller losses when the amino-acids are estimated by the copper method than when the ninhydrin method is used in their estimation. The nature of the change taking place during the hot-air drying is still obscure, but it is conceivable that, though the product formed is incapable of reacting with the ninhydrin reagent, it may still be capable of forming a complex with copper. The author has been unable to demonstrate any loss of ammonia by amino-acids when dried on filter paper in the presence of solvents under the conditions usually used in chromatographic practice. The method of amino-acid assay described in this paper has eliminated all serious losses, and whilst some means of eliminating the apparent losses in the copper method of determination is still being investigated, it seems that the more sensitive ninhydrin method will be more useful for future work.

The labour involved in the determination of amino-acids in mixtures using the ninhydrin method is considerably less than in the copper method, since the preparation and running of control chromatograms is unnecessary. If results of the highest degree of accuracy are not required, a single amino-acid may be used as the standard in the colorimetric determination of several amino-acids in an unknown mixture, for the relative colour responses per mol. of the different amino-acids have been compared and found to be reproducible. The number of separate determinations necessary can therefore be further reduced.

No attempt was made to estimate proline or hydroxyproline in this work. Two serious difficulties were found in the determination of these amino-acids by the methods described. The colours given by the amino-acids with the ninhydrin reagent were yellow or yellow-brown, and showed a maximum light absorption at a wavelength $\lambda = 440$ mμ. Even when the colours produced were determined using this wavelength and compared with those given by the other amino-acids measured at $\lambda = 570$ mμ., the colour response per mol. of proline was less than one-third and of hydroxyproline about one-sixth of that given by leucine. Such a loss of sensitivity in the method of determination causes a corresponding increase in the errors likely to arise in the colorimetric estimation. All methods employing ninhydrin suffer from this defect with proline and hydroxyproline, but since these amino-acids form the normal type of complex with copper (2 mol. of amino-acid with 1 atom of copper), they behave in a manner identical with all the other α-amino-acids in the copper method of determination. It was also found necessary to use much larger amounts of proline and hydroxyproline before these amino-acids were visible as fluorescent spots when viewed in ultraviolet light. These two differences from the general behaviour of the α-amino-acids seriously limit the usefulness of the ninhydrin method for the determination of micro-amounts of proline and hydroxyproline.

SUMMARY

1. A ninhydrin method for the estimation of amino-acids after separation by filter-paper chromatography has been described. The applications to synthetic mixtures of pure amino-acids are given. The method uses the Moore & Stein (1948) ninhydrin reagent in a slightly modified form, and can be used in the determination of 1–30 μg. α-NH_2-N of an amino-acid.

2. Ammonia absorbed on the paper has been shown to be responsible for the high and variable values of the paper blank previously obtained in ninhydrin colorimetric methods and a means of easily removing this ammonia is described. The residual paper blank after this treatment is found to be low and constant in value for pieces of paper of equal size.

3. When chromatograms were dried in hot air at 70–100°, 20–30 % losses of α-amino-nitrogen were found for many of the amino-acids determined by the method described. When the chromatograms were dried at room temperature after removal of the solvent by washing with ether, the losses were completely eliminated and quantitative recoveries of the amino-acids made possible. This new method of drying the papers has made control chromatograms of standard amounts of amino-acids unnecessary, and greatly reduced the labour involved in amino-acid determinations.

4. No evidence of losses of amino-acid arising during the running of chromatograms due to trailing or heavy metal complex formation was found.

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The Enzymic Oxidation of Pyridoxal by Liver Aldehyde Oxidase

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Huff & Perlzweig (1944) have shown that pyridoxic acid (3-hydroxy-5-methoxy-2-methylpyridine-4-carboxylic acid) is normally present in human urine, and that the amount excreted may be increased by the intake of vitamin B_6, usually administered in the form of pyridoxine. As pyridoxal phosphate is known to be active as a coenzyme of amino-acid decarboxylases, it was reasonable to assume that the acid might be produced in the body by a direct oxidation of the aldehyde. In this paper we have been able to show that pyridoxal can, in fact, be oxidized in the presence of liver aldehyde oxidase and that the reaction product is pyridoxic acid. The course of the reaction has been followed by measuring the rate of disappearance of the aldehyde and the accompanying increase in fluorescence, and the reaction product has been isolated and identified as pyridoxic acid.

EXPERIMENTAL

Most of the enzymic studies were carried out using aldehyde oxidase (Knox, 1946) prepared from rabbit liver according to the method of Gordon, Green & Subramanyan (1940). This method was, however, far from satisfactory as the potency and stability of different preparations varied unaccountably. The enzyme seldom remained active for longer than 1 week and frequently lost its activity within a few hours after preparation. Moreover, on numerous occasions, the final $(NH_4)_2SO_4$ precipitate contained a heavy white sediment which could not be redissolved and appeared to adsorb the enzyme irreversibly, so that it was impossible to obtain an active preparation. Although it is not possible to state a definite reason, it appears that in general this occurred when the liver used was unusually heavy and light in colour. On such occasions all the soluble fractions were very turbid, and the final $(NH_4)_2SO_4$ precipitate was dense and almost colourless. Activity measurements at the different stages of the purification process have shown that in these cases the enzyme was originally present in the liver homogenate and was carried down with the $(NH_4)_2SO_4$ precipitate. One suggestion is that the difficulty in separating the enzyme might be due to the presence of large quantities of glycogen.

Because of these difficulties we have, in addition, used a preparation of aldehyde oxidase from horse liver for the isolation of the end product. This enzyme, although less fractionated than the rabbit-liver preparation, had retained its potency during 4 months' storage in the refrigerator, but the high concentration of proteins in the preparation rendered it unsuitable for reaction studies in the fluorimeter.

Crystalline pyridoxal hydrochloride (Merck) was used for the substrate solutions. A stock solution containing 10 mg./ml. remained stable for about 1 month when kept in the refrigerator without exposure to light. Fresh dilutions of this solution were prepared for each test, and all tests were carried out in tubes wrapped in tin foil to minimize the effect of light. The enzymic oxidation of pyridoxal was demonstrated by the use of three independent methods.

Decolorization of methylene blue. This method was used only in preliminary tests to show that pyridoxal undergoes oxidation in the presence of aldehyde oxidase. Determinations were carried out in evacuated Thunberg tubes which contained the substrate in 0·1 M-phosphate buffer, pH 7, in the main tube and the enzyme in the side arm. After mixing, the oxidation was followed by measuring the decolorization of the methylene blue through the decrease in absorption at 660 mμ., using the Coleman Junior spectrophotometer. No decolorization was observed in blank tubes containing no enzyme. In this way pyridoxal was shown to be oxidized in the presence of the two liver preparations mentioned. Xanthine oxidase, however, appeared to be inactive towards pyridoxal.

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